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Signature

Applicant : John A. Arcadi (Deceased) Confirmation No. 6120  
Application No. : 09/383,114  
Filed : August 25, 1999  
Title : COMPOSITION AND METHOD FOR TREATING CARCINOMA  
Grp./Div. : 1614  
Examiner : Rebecca Cook  
Docket No. : 35687/RWJ/H29

**SUBMISSION OF APPELLANT'S BRIEF (§ 1.192)  
TO THE BOARD OF PATENT APPEALS AND INTERFERENCES**

Mail Stop Appeal Brief-Patents  
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January 28, 2005

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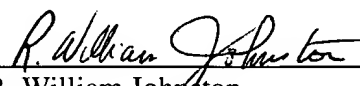
Enclosed for filing are the **original and two copies** of Appellant's Brief for this application.

- \_\_\_\_\_ An extension of time to file Appellant's Brief is requested, and a Petition for Extension of Time and the applicable fee are enclosed.
- X   Our check for \$250.00 to cover the fee for the appeal brief is enclosed.
- \_\_\_\_\_ An oral hearing of the appeal is requested, and our check for \$\_\_\_\_\_, the fee for the oral hearing, is enclosed.

The Commissioner is hereby authorized to charge any further fees under 37 CFR 1.16 and 1.17 which may be required by this paper to Deposit Account No. 03-1728. Please show our docket number with any charge or credit to our Deposit Account. **A copy of this letter is enclosed.**

Respectfully submitted,

CHRISTIE, PARKER & HALE, LLP

By   
R. William Johnston  
Reg. No. 17,968  
626/795-9900


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APPELLANT'S BRIEF

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1. REAL PARTY IN INTEREST

The real party in interest is the assignee, Huntington Medical Research Institutes, a California nonprofit public-benefit corporation.

2. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

3. STATUS OF CLAIMS

Claims 1-30 are in this application. No claim is allowed. Claims 1, 9-12, 14, 16, 20-24, 28 and 29 are rejected under 35 USC § 112, first paragraph. Claims 1-30 are rejected under 35 USC § 103(a). Claims 1-30 are attached hereto in "APPENDIX OF CLAIMS INVOLVED ON APPEAL".

**4. STATUS OF AMENDMENTS**

No amendment was filed after the Final Rejection dated 08/31/04.

**5. SUMMARY OF INVENTION**

The present invention is directed to a method for treating a patient with carcinoma, such as prostate cancer, with Rhodamine-123 (methyl o-(6-amino-3-imino-3H-xanthen-9-yl) benzoate monohydrochloride). The protocol for treating prostate cancer in accordance with this invention is described in the application on pages 11-16.

The invention also provides a solution of Rhodamine-123 for treating a patient with carcinoma. The preferred treatment solution comprises ethyl alcohol and an effective amount of Rhodamine-123 dissolved in water. The treatment solution and a stock solution of Rhodamine-123 from which treatment solution can be made are described in the application on pages 11 at lines 23-25, and page 12, line 21-page 13, line 16.

The application (on pages 11-16) sets forth in precise detail how to administer Rhodamine-123 in the treatment of patients with hormone-refractory prostate cancer, one form of carcinoma. As explained in detail below, clinical tests in accordance with that protocol showed a substantial decrease in Prostate-Specific Antigen (PSA) for 4 out of 12 patients. Moreover, for two of those patients the decrease in PSA exceeded 50%. This is important because survival time for patients with hormone-refractory prostate cancer is significantly extended if a patient has a 50% or greater drop in PSA level after treatment.

Applicant is the first to show how to use Rhodamine-123 in treating patients with one type of carcinoma. The clinical tests demonstrating the efficacy of Applicant's invention were expensive, and time-consuming. It would be unfair and unreasonable to limit the scope of applicant's claims because he did not have sufficient time and assets to demonstrate the efficacy of his treatment in clinical tests on a variety of carcinomas. The application presents a clear blueprint how to practice the invention. Those skilled in the art would have no difficulty, given sufficient time, money and available patients, in using applicant's invention to treat patients with other types of carcinoma without any undue experimentation. Clinical tests performed in accordance with applicant's protocol set forth in the application have demonstrated that Rhodamine-123 can be administered in effective amounts without causing toxicity. Those

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skilled in the art would not have to experiment unduly to adjust the protocol as may be necessary to accommodate patients with different types of carcinoma.

**6. ISSUES ON APPEAL**

**I.** Whether claims 1, 20-24, 28 and 29 are supported by enabling disclosure as required by 35 U.S.C. § 112, first paragraph.

**II.** Whether claims 9-12, 14 and 16 are supported by enabling disclosure as required by 35 U.S.C. § 112, first paragraph.

**III.** Whether claims 1-30 are patentable (under 35 U.S.C. § 103(a)) over Arcadi (1986) or Arcadi (1990) in view of US Patent No. 6,880,141 (Tang, et al.) or EMBASE 94148842 and further in view of MEDLINE AN 93172422.

**7. GROUPING OF CLAIMS**

**Group I** Claims 29 and 30 are directed to treating a patient with carcinoma or prostate cancer (one form of carcinoma) by administering Rhodamine-123 to the patient in an amount sufficient to effect *in vivo* destruction of carcinoma cells (claim 29) or prostate cancer cells (claim 30).

**Group II** Claims 1 and 28 define a method for treating a patient with carcinoma by intravenous administration of a solution of Rhodamine-123 in ethyl alcohol and water in an amount sufficient to effect *in vivo* destruction of prostate cancer cells (claim 1) or carcinoma cells (claim 28).

**Group III** Claims 2, 3, 4, 5, 6, 7, 8, 17, 18 and 19 are directed to a method for treating a patient with prostate cancer by administration of Rhodamine-123 to the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, including the steps of measuring the patient's PSA (prostate specific antigen) level before and after treatment to confirm the destruction of prostate cancer cells in the patient. The use of PSA measurements is described in the application on page 2, lines 15-20; page 12, lines 15-20; and page 16, lines 12-26.

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**Group IV** Claims 9, 10, 11, 12, 13, 20, 21, 22, 23, and 24 define a treating solution for treating a patient with carcinoma. The solution comprises ethyl alcohol and an effective amount of Rhodamine-123 dissolved in water.

**Group V** Claims 14, 15 and 16 define a stock solution for preparing a treating or administration solution for treating carcinoma. The stock solution comprises Rhodamine-123 dissolved in ethyl alcohol.

**Group VI** Claims 25, 26 and 27 cover treating a prostate cancer patient with Rhodamine-123 in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, and measuring the patient's prostate specific acid phosphatase level before and after treatment to confirm the destruction of prostate cancer cells in the patient. The use of prostate specific acid phosphate levels is described in the application on page 2, lines 15-20; and page 14, line 28-page 16, line 10.

**8. ARGUMENT**

**I. CLAIMS REJECTED UNDER 35 § 112, FIRST PARAGRAPH**

A. Claims 1, 20-24, 28 and 29 were rejected under 35 U.S.C. § 112, first paragraph, "because the specification, while being enabling for using Rhodamine-123 to treat prostate cancer, does not reasonably provide enablement for treating any and all carcinomas." This rejection makes an unwarranted assumption, and it would apparently impose on the Applicant the expensive, time-consuming, and unnecessary burden of providing evidence from human clinical trials to show the efficacy of Rhodamine-123 for treating other types of carcinomas in addition to the clinical evidence submitted (in the application and during prosecution) for treating prostate cancer.

The application states (page 1, line 24, Rhodamine-123 "is selectively toxic for carcinoma cells.") Moreover, the Declaration (attached to this brief as Exhibit A) dated July 22, 2002 by Dr. Lawrence W. Jones, and filed with Applicant's amendment filed July 22, 2002, explains in paragraph 4 "carcinoma is any of the various types of malignant tumor derived from epithelial tissue, including cancer of the breast, liver, pancreas, bladder, lung, skin, and colon, as well as other organs of the human body. See Stedman's Medical Dictionary, 24<sup>th</sup> Edition, Published 1982, page 223, which is attached as Exhibit 'A'."

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The July 22, 2002 Declaration of Dr. Jones further explains in paragraphs 5 and 6 that:

"5. The patent application, as originally filed, states on page 1, line 24 that 'Rh-123 is selectively toxic for carcinoma cells.' Accordingly, the patent application as originally filed makes clear to ordinary workers in this field that rhodamine-123 is useful for treating carcinoma, such as prostate cancer.

6. The application also makes it clear how to administer rhodamine-123 safely to patients, so the efficacy of the drug for treating all types of carcinoma can be determined by routine experiments."

As explained in a Declaration dated February 23, 2001 by Dr. Jones, and filed with Applicant's amendment filed the same date, he is the physician who provided supervision as Principal Investigator for the experimental testing of Rhodamine-123 to treat human prostate cancer *in vivo*. See paragraph 2 of his February 23, 2001 Declaration (attached hereto as Exhibit B).

The attached 2/23/01 Declaration (Exhibit B) of Dr. Jones, the Director of Prostate Research Program at Huntington Medical Research Institutes (HMRI), the assignee of this application, shows (paragraphs 2-8) that even very limited clinical trials to the date of the Declaration produced a substantial decrease in PSA measurements for 6 out of 12 volunteers, and that for 2 of the volunteers, the drop exceeded 50%, which has been reported by an independent source as indicating that survival time for those two patients will be significantly extended. See the reference referred to in the attached Declaration, namely, the Kantoff, P.W., et al. article in the *Journal of Clinical Oncology*, Vol. 17, No. 8 (August), 1999:pp 2506-2513, at page 2509.

The Manual of Patent Examining Procedure (Rev. 2, May 2004), at Section 2107.03(I), states, "The applicant does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty, nor

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does he or she have to provide actual evidence of success in treating humans where such a utility is asserted. Instead, as the courts have repeatedly held, all that is required is a reasonable correlation between the activity and the asserted use. *Nelson v. Bowler*, 626 F.2d 853, 857, 206 U.S.P.Q. 881, 884 (C.C.P.A. 1980)." [At page 2100-43] Accordingly, Applicant submits that the Declarations of Dr. Jones establish a reasonable correlation between the demonstrated activity of Rhodamine-123 on prostate cancer in human patients, and the asserted use of Rhodamine-123 for treating other forms of carcinoma, i.e., a malignant tumor derived from epithelial tissue.

**B.** Claims 9-12, 14 and 16 were rejected under 35 U.S.C. § 112, first paragraph, "because the specification, while being enabling for the amounts of ethyl alcohol recited in claims 13 and 15, does not reasonably provide enablement for any and all amounts of ethyl alcohol. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims."

The application on page 2, lines 29-32 states:

"The invention also provides a stock solution for preparing an administration solution used in treating prostate cancer. The stock solution comprises Rh-123 dissolved in ethyl alcohol (preferably 95% ethyl alcohol and 5% water). The concentration of the Rh-123 in the stock solution is between about 5 and about 25 mg per ml."

The application on page 2, lines 25-28, describe a treating solution which "comprises ethyl alcohol and Rh-123 dissolved in water."

Further, the application at page 11, lines 23-25 (under the heading "**PROTOCOL**") refers to the "Stock Solution" and a "Final Preparation."

In addition, the application on page 12, line 21 through page 13, line 32 describes a number of examples of how a treatment solution is prepared from the stock solution. For example, page 12, lines 21-30, explains in detail how to prepare a treatment solution for a patient weighing 70kg from the stock solution. In addition to the specific treatment solution, that

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description states "Additional ethyl alcohol can be added up to a total of about 5% by volume to ensure that the Rh-123 stays in solution." Applicant agrees with the Examiner's statement in the Office Action dated 03/05/2004 on page 5 that "The level of ordinary skill in the pharmaceutical art is high, generally that of a PHD or MD." Accordingly, applicant's disclosure enables any person skilled in the art to use the invention commensurate with the scope of the claims by simply using a treating solution which contains an effective amount of Rhodamine-123 and sufficient ethyl alcohol to ensure that the Rhodamine-123 stays in solution. This is supported by the July 22, 2002 Declaration (Exhibit A herein) of Dr. Jones in paragraph 6, namely, "The Application also makes it clear how to administer rhodamine-123 safely to patients, so that the efficacy of the drug for treating all types of carcinoma can be determined by routine experiments."

Applicant is entitled to the more generic claims because it has long been the law that "A specification may, within the meaning of 35 USC § 112, paragraph one, contain a written description of a broadly claimed invention without describing all species that claim encompasses." *Utter v. Hiraga*, 845 F.2d 993 (Fed. Cir. 1988). The claims should not be rejected because of "overbreadth". "[T]hat word . . . has long ago been discredited as a basis for determining sufficiency of a specification". See *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369, 58 C.C.P.A. 1069 (1971), which held that the Patent Office should be concerned with support or non-support of a generic term, not its breadth.

In the present case, the claims defining the treatment of carcinoma, and those defining the solutions useful in treating carcinoma are clearly supported by the disclosure and the application. In view of the above explanation, claims 1, 9-12, 14, 16, 20-24, 28 and 29 comply with 35 USC § 112, first paragraph.

## **II. CLAIMS REJECTED UNDER 35 USC § 103(a)**

In the Office action dated 08/31/04, on page 3, claims 1-30 are "rejected under 35 USC § 103(a) as being unpatentable over Arcadi in view of 5,880,141 (Tang, et al.) or EMBASE 94148842 and further in view of MEDLINE AN 93172422 for the reasons given in the Paper of March 5, 2004." There are two Arcadi references of record. One was published in 1986, the other in 1990. The 08/31/04 Office action probably refers to the 1990 Arcadi article appearing in



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*Journal of Surgical Oncology* 44:103-108 because that is the only Arcadi reference cited in the Office Action dated 03/05/2004. However, the Office Action dated 08/31/2004 on page 3, 5 lines from the bottom, refers to "Rhodamine-123 in saline solution." The Arcadi (1990) reference does not refer to a saline solution. However, a saline "suspension" of Rhodamine-123 is referred to in the 1986 Arcadi reference appearing in "*UROLOGY*", Volume XXVIII, Number 6, at page 502. The 1990 Arcadi reference discloses (at page 104) "a 50% DMSO solution containing 5 mg/ml of rhodamine 123." For clarity, the two Arcadi references will be referred to as Arcadi (1986) and Arcadi (1990). Incidentally, the Applicant, now deceased, is the author of both Arcadi references.

In responding to the rejection under 35 U.S.C. § 103(a), Applicant will assume that the rejection of claims 1-30 relies on both Arcadi references. Those two references were previously cited and overcome, resulting in allowance of claims 1-8, 17-19, 25-27 and 30 in the Office Action dated 07/02/2003, when Examiner J. Goldberg had the application.

For the reasons stated below, taken in conjunction with the application, declarations, and prior art, all of record, claims 1-30 are patentable over either of the two Arcadi references, even in view of the secondary prior art, namely, U.S. Patent No. 5,880,141 (Tang, et al.), EMBASE 94148842, and MEDLINE AN 93172422, which were first cited in the 03/05/2004 Office action by the Examiner presently in charge of the application.

In addition to the two Jones declarations dated July 22, 2002 and February 23, 2001 (Exhibits A and B, respectively), a third Declaration (dated October 30, 2001) by Dr. Jones is attached hereto as Exhibit C, and Applicant's Declaration dated February 13, 1997 is attached hereto as Exhibit D. The four Declarations, the application, and prior art, all of record, establish the following points refuting the rejection of claims 1-30:

1. Long-felt need -- Prostate cancer accounts for about 40,000 deaths annually, and there has been no satisfactory treatment for it. See the application, page 1, lines 15-16, and Exhibit C, Jones 10/30/01 Declaration, paragraphs 1 and 2.
2. Although it was known at least as early as 1982 that Rhodamine-123 could reduce the clonal growth of carcinoma of cells *in vitro*, the compound was still dismissed as inadequate for clinical development.

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3. The two Arcadi references of 1986 and 1990 do not provide one skilled in the art with a reasonable expectation that Rhodamine-123 would be any more effective in combating prostate cancer than any of the hundreds of other drugs tested *in vitro* or in laboratory animals as potential anti-tumor agents, but which subsequently failed in clinical tests, or never reached that stage.

4. Neither the saline suspension of Rhodamine-123 disclosed in Arcadi (1986) nor the solution of Rhodamine-123 in DMSO disclosed in Arcadi (1990) would be acceptable for clinical trials. See Applicant's declaration attached hereto as Exhibit D.

5. Nothing in the secondary references relied on in rejecting claims 1-30 makes up for the deficiency in the primary references of Arcadi (1986) and Arcadi (1990).

At least as early as 1982, Rhodamine-123 was known to reduce the clonal growth of carcinoma in cells *in vitro*. Nevertheless, even after the two Arcadi references published in 1986 and 1990 reporting encouraging *in vitro* and animal experiments from using Rhodamine-123 to damage prostate cancer cells, there was still substantial professional skepticism about Rhodamine-123 for treating human prostate cancer. See for example the article entitled "Synthesis and Evaluation of Novel Rhodacyanine Dyes That Exhibit Anti-Tumor Activity" by Kawakami, et al., published in Journal of Medical Chemistry in 1997 at 40, 3151-3160 (attached to Exhibit "C" herein as "Exhibit "B)"). On page one of that article, the authors, referring to various organic compounds, including Rhodamine-123, which had been considered as potential anti-tumor drugs, state that: "In spite of high potential as antitumor agents, none of them have met the criteria for clinical development, such as water solubility, stability, toxicity, and pharmacokinetics".

The Declaration (Exhibit C herein) executed 10/30/01 by Lawrence W. Jones, M.D., also explains why references such as Arcadi (1986 and 1990) do not provide one skilled in this art with a reasonable expectation that rhodamine-123 would be effective to combat said prostatic cancer in human. As stated in the 10/30/01 Declaration (paragraphs 4 and 5), Dr. Jones, as Principal Investigator, supervised clinical testing of rhodamine-123 under Phase I of protocol approved by the FDA. Paragraphs 6-11 of the 10/30/01 Declaration state:

“6. Over the past 50 years, hundreds of drugs tested *in vitro* and in laboratory animals have shown potential as antitumor agents, but subsequently failed in clinical tests, or never reached that stage. For example, see U.S. Patent 5,360,803 (filed November 6, 1992, and assigned to Dan Farber Cancer Institute and Fuji Photo Film Co., Ltd.), which discloses at least 348 antitumor agents for possible treatment of prostate cancer in humans. I review regularly publications and reports dealing with agents for treating prostate cancer and many other forms of carcinoma. As far as I am aware, none of those disclosed by that patent have been accepted by the medical profession as a treatment for prolonging life of patients afflicted with hormone-refractory prostate cancer.

7. Contrary to the statement in the 03/11/2002 and 10/31/2002 Office actions, the 1983 Bernal et al. reference referred to in paragraph 3 above would not cause one of ordinary skill in this work to reasonably expect that rhodamine-123 would be any more effective in combating carcinoma than any of many other drugs which have been tested *in vitro* and in laboratory animals with promising results, but which have failed to produce any therapeutic effects in human patients. Nothing in the Bernal et al. reference discloses that human life can be prolonged by treating victims of carcinoma with rhodamine-123.

8. An important problem in the management of carcinoma is the heterogeneity of the disease. For example, unequivocal evidence from animal studies, from the growth of human prostate cancer in tissue culture, in the xenograft system, and from human biopsy material shows that many different types of tumor cells exist within prostate cancer, and in many other

forms of carcinoma. Accordingly, even though a drug may be demonstrated to have anticarcinoma activity in laboratory *in vitro* and animal experiments, that does not justify a reasonable expectation that it will be effective in treating human carcinoma. This is well recognized by skilled workers in this field. For example, attached to this Declaration as Exhibit A is a paper by the inventor and others (including me) entitled Studies of Rhodamine-123: Effect on Rat Prostate Cancer and Human Prostate Cancer Cells In Vitro, presented in the *Journal of Surgical Oncology* 59:86-93 (1995), which describes some experimental work providing some basis for this patent application. Under Editorial Comments at the end of the paper, Dr. T. Vincent Shankey, with the Departments of Urology and Pathology at Loyola University Medical Center in Maywood, Illinois, rejects the work described in the paper as supporting the thesis that Rh-123 may be an effective agent for the treatment of metastatic hormone-refractory prostate cancer because it is a connection that has too often failed in the past. Dr. Shankey's criticism cites other authorities who have pointed out that the local environment of solid malignancies *in situ* has a profound impact on the responsiveness or nonresponsiveness of cancers where they really count to a patient in his or her body.

9. Further evidence of skepticism about rhodamine-123 long after the 1983 Bernal et al. reference appears in an article entitled Synthesis and Evaluation of Novel Rhodacyanine Dyes That Exhibit Anti-Tumor Activity by Kawakami et al., published in the *Journal of Medical Chemistry* in 1997 at 40, 3151-3160 (copy attached as Exhibit B). On page 1 of that article, the authors, referring to various organic compounds, including rhodamine-123, which have been explored as potential antitumor drugs, state that:

In spite of high potential as antitumor agents, none of them have met the criteria for clinical development, such as water solubility, stability, toxicity, and pharmacokinetics.

10. At least as early as 1982, rhodamine-123 was known to reduce the clonal growth of carcinoma cells *in vitro*. For example, see attached Exhibit C, an article entitled Rhodamine-123 Selectively Reduces Clonal Growth of Carcinoma Cells *In Vitro*, published in *Science*, Vol. 218, pp. 1117 & 1118, (10 December 1982). Even so, there was still substantial professional skepticism about the efficacy of rhodamine-123 for treating human carcinoma. For example, as late as 1997, workers at Fuji Photo Film Co., Ltd. and Harvard Medical School dismissed rhodamine-123 as failing to meet the necessary criteria for clinical development (see Exhibit B referred to above in paragraph 9).

11. Based on my experience and the skepticism of colleagues with respect to the possible efficacy of rhodamine-123 for treating carcinoma, the 1983 Bernal et al. reference does not provide a reasonable expectation that rhodamine-123 would be any more effective for treating human carcinoma than any of many other agents which showed promising laboratory results, and failed to be therapeutic. The drug industry and the medical profession have spent millions of dollars and thousands of research hours seeking effective therapy for various forms of carcinoma. By any objective standard, if the Bernal et al. reference had actually created a reasonable expectation that treatment with rhodamine-123 would prolong the life of carcinoma victims, the compound would have been put to wide use instead of being dismissed as clinically inadequate by other workers in that field.”

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The quoted comments by Dr. Jones dealing with the Bernal et al. reference apply equally as well to the two Arcadi references. The first Examiner (J. Goldberg) assigned to this application cited the Bernal et al. and the Arcadi references in rejecting the claims which he eventually allowed in the 07/02/2003 Office action referred to above. The present Examiner relied on the Arcadi and Bernal et al. references in rejecting all claims in the 03/05/2004 Office action, but in the 08/31/2004 Office action dropped the Bernal et al. reference because "Bernal is considered cumulative and is no longer applied."

Applicant's Declaration (Exhibit D) executed February 13, 1997, and submitted with an Amendment dated February 14, 1997 in parent Application No. 08/516,004, filed August 16, 1995, provides evidence that his two prior publications (the Arcadi 1986 and 1990 references) do not make his claimed invention obvious. His declaration explains why the saline suspension of Rhodamine-123 disclosed in his 1986 article and the dimethylsulfoxide (DMSO) solution of Rhodamine-123 described in his 1990 article would not be acceptable for treating patients. The saline suspension would result in uncertain dosage because of the limited solubility of Rhodamine-123 in that suspension. The DMSO solution was unacceptable because it killed some of the mice on which was tested. The Declaration also states that none of the mice died when injected with any of the ethyl alcohol/glucose solutions used by the inventor for his experimental work. For convenience of reference, paragraphs 3-5 of the Applicant's Declaration (Exhibit D) are set forth below.

"3. My 1986 article discloses the testing of a saline suspension of rhodamine-123 (Rh-123) on rats which had been implanted subcutaneously in the flanks with transplantable R3327-H Dunning rat prostate adenocarcinoma. For that test I wanted to use a solution of 5 mg. of Rh-123 per ml. of solution, but the Rh-123 was in the form of crystalline particles, which would not dissolve entirely in the saline solvent. Accordingly, I stirred the saline suspension vigorously to suspend the Rh-123 particles, filled a hypodermic syringe with the appropriate amount of saline

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suspension (which contained dissolved Rh-123 and undissolved particles of Rh-123 in the amount of 5 mg. of Rh-123 per ml. of suspension), and within five to ten seconds injected the suspended Rh-123 into the rat being treated. The subcutaneous injection of the saline suspension of Rh-123 described in my 1986 article would be unacceptable for treating patients because the suspension would result in uncertain dosage, and there would be an unknown amount of solubilizing of the Rh-123.

4. To overcome the problem of using Rh-123 in a saline suspension, I tried an experiment in which I dissolved the Rh-123 in a solution containing 50% dimethylsulfoxide (DMSO) in distilled water. The Rh-123 was sufficiently soluble (5 mg. Rh-123 per ml. of solvent) in the mixture of DMSO and water so as to avoid having to use a suspension. However, I later learned during the course of my work described in my 1995 article<sup>1</sup> that the 50% DMSO mixture with water was unacceptable for administering Rh-123 because it killed some of the mice tested. This is referred to in my patent application on page 6 beginning at line 25 under the section entitled "Toxicity Studies on Mice". Incidentally, in reviewing the description in the application under that heading, I noticed an error in reporting my work. The sentence which begins on page 6, line 29 should read as follows:

"For each solvent there were five groups of five mice each, with the Rh-123 dose per group being 0, 2.0, 7.5, 15, and 20 mg/kg of body weight."

The following sentence should have been inserted after the one set forth above:

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<sup>1</sup> Attached as Exhibit A to Exhibit C herein.

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“A sixth group of five mice were not given any solvent or Rh-123.”

The following table sets forth more fully and accurately the results of the toxicity study referred to in my patent application:

<b>Group No.</b>	<b>No. of Deaths</b>	<b>Treatment</b>
Group 1a	3	20 mg/kg* Rh-123 in 50% DMSO + 50% water**
Group 1b		20 mg/kg Rh-123 in 5% alcohol + 5% glucose*** in water
Group 2a	1	15 mg/kg Rh-123 in 50% DMSO + 50% water
Group 2b		15 mg/kg Rh-123 in 5% alcohol + 5% glucose in water
Group 3a		7.5 mg/kg Rh-123 in 50% DMSO + 50% water
Group 3b		7.5 mg/kg Rh-123 in 5% alcohol + 5% glucose in water
Group 4a		2.0 mg/kg Rh-123 in 50% DMSO + 50% water
Group 4b		2.0 mg/kg Rh-123 in 5% alcohol + 5% glucose in water
Group 5a	2	50% DMSO + 50% water only
Group 5b		2.0 mg/kg Rh-123 in 50% DMSO + 50% water
Group 6		None

\*Mg. of Rh-123 per kg. of body weight

\*\*Distilled water was used for all treatments

\*\*\*The ethyl alcohol was present in the solution in the amount of 5% by volume, and the glucose 5% by weight.

Each group in the above table included five mice. As the table shows, three of the five mice in Group 1a died when injected with a dose of 20 mg/kg of Rh-123 dissolved in a mixture of 50% DMSO and 50% distilled water at a concentration of 5 mg. Rh-123 per ml. of solution. One of the five mice in Group 2a died when injected with 15 mg/kg Rh-123 dissolved in a solution of 50% DMSO and 50% distilled water by volume at a concentration of 5 mg. of Rh-123 per ml. of solution.



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Two of the five mice in Group 5a died when injected with a solution of 50% DMSO and 50% distilled water containing no Rh-123.

None of the mice died when injected with any of the ethyl alcohol/glucose solutions, even at a dose of 20 mg/kg Rh-123 in a solution of 5 mg. of Rh-123 per ml. of solution, included which 5% ethyl alcohol by volume and 5% glucose by weight in distilled water.

5. The toxicity studies set forth in paragraph 4 above show that the use of the DMSO solutions described in my 1990 article would be totally unacceptable for treating patients.”

Accordingly, nothing in the Arcadi references of 1986 and 1990 (or any of the other cited prior art) discloses, suggests, or provides any motivation for clinical use of Rhodamine-123, or the use of ethyl alcohol to make a satisfactory solution of Rhodamine-123 for treating carcinoma such as prostate cancer.

With respect to the claims of Group III, namely, claims 2-8, 17, 18 and 19, the Office action dated 08/31/2004 states on page 4 “one skilled in the art would be motivated to measure PSA levels before and after treatment because as disclosed by MEDLINE and AN 93172422, it is well-known in the management of prostate cancer to measure PSA both pre and post treatment.” This mischaracterizes the reference, which simply discloses that between 1986 and 1989 117 prostate cancer patients received “external beam radiotherapy”. Thereafter, PSA values were measured to identify “biochemical relapse”, which was “defined as an increasing prostate specific antigen level after treatment”. The PSA measurements were not used “in the management of prostate cancer”. Instead, the measurements were merely used to determine when there was a biochemical relapse. Nothing in the cited reference would make it obvious to use PSA measurements to confirm the destruction of prostate cancer cells in a patient treated chemically with Rhodamine-123. Thus, the rejection of these claims is based on unsupported conjecture.

## Application No. 09/383,114

The Office action dated 08/31/2004 on page 3 and 4 appears to reject applicant's claims which deal with Rhodamine-123 dissolved in ethyl alcohol. Applicant believes those claims covered by that rejection are claims 1 and 28 (Group II), claims 9-13, 20-24 (Group IV) and Claims 14, 15 and 16 (Group V). In any event, the rejection states "Arcadi discloses Rhodamine-123 in saline solution<sup>2</sup> (Rh-123) is effective against prostate cancer. Arcadi does not disclose a composition comprising Rh-123 and ethyl alcohol and sugar. However, Tang discloses that a solvent system comprising ethanol and dextrose (D5W) is known in the pharmaceutical art and that it produces low toxicity upon systemic administration. Furthermore, EMBASE 9414882 discloses that ethanol enhances drug solubility. One of ordinary skill in the art would be motivated to use a composition comprising Rh-123 and ethanol because EMBASE 9414882 discloses that ethanol enhances drug solubility. One would further be motivated to use sugar because Tang discloses that ethanol and D5W produces low toxicity upon systemic administration."

The claims in question are patentable because the only "motivation" for combining the references comes from Applicant's disclosure. There is not the slightest suggestion in either Tang or EMBASE 9414882 that the solution in either would be useful with Rhodamine-123. Moreover, nothing in the Arcadi references points to the use of the solutions disclosed in the other references. There is no teaching or motivation in either the primary or cited references for combining them to achieve Applicant's invention defined by the claims, and the Office action does not identify any reason for such combination. Instead, the Office action uses Applicant's own disclosure as a blueprint to pick and choose from voluminous prior art an unlikely combination of references, which would not occur to anyone of ordinary skill in the art, absent Applicant's disclosure.

The rejection enters the "tempting but forbidden zone of hindsight" condemned by the U.S. Court of Appeals for the Federal Circuit in reversing the Board of Patent Appeals and Interferences in the case of *In re Dembiczak*, 50 U.S.P.Q. 2d 1614, at p. 1616. As stated by the *Dembiczak* Court, "Measuring a claimed invention against the standard established by Section

---

<sup>2</sup> As explained above, neither Arcadi reference discloses a saline "solution" of Rh-123. Instead, Arcadi (1986) discloses Rh-123 in a saline suspension, which is unacceptable for human treatment.

**Application No. 09/383,114**

103 requires the oft-difficult but critical step of casting the mind back to the time of invention, to consider the thinking of one of ordinary skill in the art, guided only by the prior art references and the then-accepted wisdom in the field." (at page 1617) The Court further stated that:

"the best defense against the subtle but powerful attraction of a hindsight-based obviousness analysis is rigorous application of the requirement for a showing of the teaching or motivation to combine prior art references."

\* \* \*

"Combining prior art references without evidence of such a suggestion, teaching or motivation simply takes the inventor's disclosure as a blueprint for piecing together the prior art to defeat patentability — the essence of hindsight."

As the *Dembiczak* Court stated (at page 1617) "the Board must identify specifically . . . the reasons one of ordinary skill in the art would have been motivated to select the references and combine them". The Office action does not identify any reason for such combination.

**9. CONCLUSION**

The Examiner has not set forth a *prima facie* case that the pending claims are obvious in view of the cited art. The rejection of the claims should be reversed.

Respectfully submitted,

CHRISTIE, PARKER & HALE, LLP

By R. William Johnston  
R. William Johnston  
Reg. No. 17,968  
626/795-9900

Encls.: Appendix Of Claims Involved In The Appeal  
Exhibits A, B, C, And D.

RWJ/cls  
CLS PAS604511.1-\*01/28/05 2:21 PM



## APPENDIX OF CLAIMS INVOLVED ON APPEAL

1. (Previously Presented) A method for treating a patient with carcinoma comprising intravenous administration of a solution of Rhodamine-123 in ethyl alcohol and water in an amount sufficient to effect *in vivo* destruction of prostate cancer cells.

2. (Original) A method for treating a patient with prostate cancer and having a PSA level above about 5, the method comprising measuring the PSA level in the blood of the patient, administering Rhodamine-123 to the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, and thereafter measuring the patient's PSA level to confirm the destruction of prostate cancer cells in the patient.

3. (Previously presented) The method according to claim 2 which includes the step of measuring the patient's PSA level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of PSA in the blood of the patient.

4. (Previously presented) The method according to claim 1, 2 or 3 which includes injecting the patient with about 250 ml of a solution containing Rhodamine-123.

5. (Previously presented) The method according to claim 1, 2, or 3 in which the administration of Rhodamine-123 is completed within about four hours.

**Application No. 09/383,114**

6. (Previously presented) The method according to claim 1, 2, or 3 in which the patient is treated with up to about 30 mg Rhodamine-123 per kg of body weight every other day.

7. (Previously presented) The method according to claim 1, 2, or 3 in which the patient is treated with between about 0.2 and about 15 mg of Rhodamine-123 per kg of patient body weight.

8. (Previously presented) The method according to claim 1, 2, or 3 in which the patient is administered the solution of Rhodamine-123 at intervals of at least 24 hours, and in increasing amounts until the patient exhibits evidence of toxicity due to the Rhodamine-123, and thereafter administering Rhodamine-123 to the patient in an amount and at a rate less than that which causes toxicity.

9. (Previously Presented) A solution for treating a patient with carcinoma, the solution comprising ethyl alcohol and an effective amount of Rhodamine-123 dissolved in water.

10. (Previously presented) The solution according to claim 9 which includes dissolved sugar susceptible to metabolic assimilation.

11. (Previously presented) The solution according to claim 10 in which the sugar is selected from the group consisting of dextrose, glucose, and fructose.

12. (Previously presented) The solution according to claim 10 or 11 in which the sugar is present by an amount equal to about 5% by weight.

**Application No. 09/383,114**

13. (Previously presented) The solution according to claim 9, 10, or 11 in which the ethyl alcohol is present in an amount between about 0.2% and about 5% by volume.

14. (Previously Presented) A stock solution for preparing an administration solution for treating carcinoma, the stock solution comprising Rhodamine-123 dissolved in ethyl alcohol.

15. (Previously presented) The stock solution according to claim 14 in which the solution contains about 95% ethyl alcohol by volume and about 5% sterile water by volume.

16. (Previously presented) The solution according to claim 14 or 15 in which the Rhodamine-123 is present in an amount between about 4 and about 25 mg/ml of solution.

17. (Original) A method for treating a patient with prostate cancer and having a PSA level above about 5, the method comprising oral administration of Rhodamine-123 in a pill which releases the Rhodamine-123 for absorption by the patient, and in an amount sufficient to effect *in vivo* destruction of prostate cancer cells in the patient, measuring the patient's PSA level after treatment, and thereafter administering Rhodamine-123 to the patient at a rate sufficient to substantially decrease the patient's PSA level.

18. (Previously presented) The method according to claim 17 in which the pill releases between about 0.2 and about 30 mg of Rhodamine-123 per kg of patient body weight.

**Application No. 09/383,114**

19. (Previously presented) The method according to claim 17 or 18 in which the Rhodamine-123 is released within between about 2 and about 24 hours.

20. (Previously Presented) A method for prolonging human life of a patient with carcinoma, the method comprising treating the patient by dissolving Rhodamine-123 in a solvent which includes ethyl alcohol to form a stock solution, diluting the stock solution with water to form a treatment solution which includes Rhodamine-123, water and ethyl alcohol, and administering the treatment solution to the patient in an amount sufficient to effect *in vivo* destruction of carcinoma cells.

21. (Previously presented) The method according to claim 20 which includes the step of measuring the patient's PSA level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of PSA in the blood of the patient.

22. (Previously presented) The method according to claim 20 or 21 which includes injecting the treatment solution intravenously.

23. (Previously presented) The method according to claim 20 or 21 in which the stock solution contains between about 4 and about 25 mg of Rhodamine-123 per liter.

24. (Previously presented) The method according to claims 20 or 21 in which the treatment solution contains between about 0.2% and about 5% ethyl alcohol by volume.

**Application No. 09/383,114**

25. (Original) A method for treating a patient with prostate cancer and having a PSA level above about 5, the method comprising measuring the prostate specific acid phosphatase level in the blood of the patient, administering Rhodamine-123 to the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, and thereafter measuring the patient's prostate specific acid phosphatase level to confirm the destruction of prostate cancer cells in the patient.

26. (Previously presented) The method according to claim 25 which includes the step of measuring the patient's prostate specific acid phosphatase level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of prostate specific acid phosphatase in the blood of the patient.

27. (Original) A method for treating a patient with prostate cancer comprising dissolving Rhodamine-123 in a solvent which includes ethyl alcohol to form a stock solution, diluting the stock with water to form a treatment solution which includes Rhodamine-123, water and ethyl alcohol, administering the treatment solution to the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, measuring the patient's prostate specific acid phosphatase level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of prostate specific acid phosphatase in the blood of the patient.

28. (Previously presented) The method for prolonging human life of a patient with carcinoma, the method comprising intravenous administration of a solution of Rhodamine-123 in



**Application No. 09/383,114**

ethyl alcohol and water in an amount sufficient to effect *in vivo* destruction of carcinoma cells.

29. (Previously Presented) A method for prolonging human life of a patient with carcinoma, the method comprising administering Rhodamine-123 to the patient in an amount sufficient to effect *in vivo* destruction of carcinoma cells.

30. (Previously Presented) A method for treating a patient with prostate cancer, the method comprising administering Rhodamine-123 to the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells.

RWJ/cls

CLS PAS567948.1-\*01/28/05 12:52 PM

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

*I hereby certify that this correspondence is being deposited with the U.S. Postal Service as first class mail in an envelope addressed to Commissioner of Patents and Trademarks, Washington, D.C. 20231 on July 25, 2002.*

Signature

Applicant : John A. Arcadi  
Application No. : 09/383,114  
Filed : August 25, 1999  
Title : COMPOSITION AND METHOD FOR  
TREATING CARCINOMA  
  
Grp./Div. : 1614  
Examiner : J. Goldberg  
  
Docket No. : 35687/RWJ/H29

Applicant : John A. Arcadi  
Application No. : 09/383,114  
Filed : August 25, 1999  
Title : COMPOSITION AND METHOD FOR  
TREATING CARCINOMA  
  
Grp./Div. : 1614  
Examiner : J. Goldberg  
  
Docket No. : 35687/RWJ/H29

**AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 20231

Commissioner:

Commissioner:

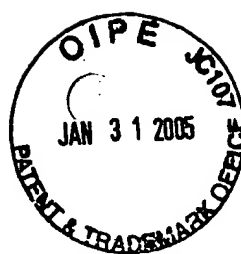
In response to the Office action dated 03/11/2002, please amend this application as follows:

**In the Claims:**

Please add new claim 28.

1. A method for treating a patient with carcinoma comprising intravenous administration of a solution of Rhodamine-123 in ethyl alcohol and water in an amount sufficient to effect *in vivo* destruction of prostate cancer cells.

2. A method for treating a patient with prostate cancer and having a PSA level above about 5, the method comprising measuring the PSA level in the blood of the patient, administering Rhodamine-123 to



the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, and thereafter measuring the patient's PSA level to confirm the destruction of prostate cancer cells in the patient.

3. A method according to claim 2 which includes the step of measuring the patient's PSA level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of PSA in the blood of the patient.

4. A method according to claim 1, 2 or 3 which includes injecting the solution in a volume of about 250 ml.

5. A method according to claim 1, 2, or 3 in which the administration of Rhodamine-123 is completed within about four hours.

6. A method according to claim 1, 2, or 3 in which the patient is treated with up to about 30 mg Rhodamine-123 per kg of body weight every other day.

7. A method according to claim 1, 2, or 3 in which the patient is treated with between about 0.2 and about 15 mg of Rhodamine-123 per kg of patient body weight.

8. A method according to claim 1, 2, or 3 in which the patient is administered the solution of Rhodamine-123 at intervals of at least 24 hours, and in increasing amounts until the patient exhibits evidence of toxicity due to the Rhodamine-123, and thereafter administering Rhodamine-123 to the patient in an amount and at a rate less than that which causes toxicity.

9. A solution for treating a patient with carcinoma, the solution comprising ethyl alcohol and an effective amount of Rhodamine-123 dissolved in water.

10. A solution according to claim 9 which includes dissolved sugar susceptible to metabolic assimilation.

11. A solution according to claim 10 in which the sugar is selected from the group consisting of dextrose, glucose, and fructose.

12. A solution according to claim 10 or 11 in which the sugar is present by an amount equal to about 5% by weight.

13. A solution according to claim 9, 10, or 11 in which the ethyl alcohol is present in an amount between about 0.2% and about 5% by volume.

14. A stock solution for preparing an administration solution for treating carcinoma, the stock solution comprising Rhodamine-123 dissolved in ethyl alcohol.

15. A stock solution according to claim 14 in which the solution contains about 95% ethyl alcohol by volume and about 5% sterile water by volume.

16. A solution according to claim 14 or 15 in which the Rhodamine-123 is present in an amount between about 4 and about 25 mg/ml of solution.

17. A method for treating a patient with prostate cancer and having a PSA level above about 5, the method comprising oral administration of Rhodamine-123 in a pill which releases the

Rhodamine-123 for absorption by the patient, and in an amount sufficient to effect *in vivo* destruction of prostate cancer cells in the patient, measuring the patient's PSA level after treatment, and thereafter administering Rhodamine-123 to the patient at a rate sufficient to substantially decrease the patient's PSA level.

18. A method according to claim 17 in which the pill releases between about 0.2 and about 30 mg of Rhodamine-123 per kg of patient body weight.

*addition*  
19. A method according to claim 17 or 18 in which the Rhodamine-123 is released within between about 2 and about 24 hours.

*reg.*  
20. A method for treating a patient with carcinoma comprising dissolving Rhodamine-123 in a solvent which includes ethyl alcohol to form a stock solution, diluting the stock with water to form a treatment solution which includes Rhodamine-123, water and ethyl alcohol, and administering the treatment solution to the patient in an amount sufficient to effect *in vivo* destruction of carcinoma cells.

*reg. on 3543C (12) then, on Bernard et al (in cl. 20 + 22-24)*  
21. A method according to claim 20 which includes the step of measuring the patient's PSA level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of PSA in the blood of the patient.

22. A method according to claim 20 or 21 which includes injecting the treatment solution intravenously.

23. A method according to claim 20 or 21 in which the stock solution contains between about 4 and about 25 mg of Rhodamine-123 per liter.

24. A method according to claims 20 or 21 in which the treatment solution contains between about 0.2% and about 5% ethyl alcohol by volume.

25. A method for treating a patient with prostate cancer and having a PSA level above about 5, the method comprising measuring the prostate specific acid phosphatase level in the blood of the patient, administering Rhodamine-123 to the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, and thereafter measuring the patient's prostate specific acid phosphatase level to confirm the destruction of prostate cancer cells in the patient.

26. A method according to claim 25 which includes the step of measuring the patient's prostate specific acid phosphatase level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of prostate specific acid phosphatase in the blood of the patient.

27. A method for treating a patient with prostate cancer comprising dissolving Rhodamine-123 in a solvent which includes ethyl alcohol to form a stock solution, diluting the stock with water to form a treatment solution which includes Rhodamine-123, water and ethyl alcohol, administering the treatment solution to the patient in an amount sufficient to effect *in vivo* destruction of prostate cancer cells, measuring the patient's prostate specific acid phosphatase level before and after treatment, and administering sufficient Rhodamine-123 to substantially decrease the level of prostate specific acid phosphatase in the blood of the patient.

28. (New) A method for treating a patient with carcinoma comprising intravenous administration of a solution of Rhodamine-123

in ethyl alcohol and water in an amount sufficient to effect *in vivo* destruction of carcinoma cells.

#### REMARKS

Reconsideration of this application is requested.

Claims 1-8, 17-19, and 25-27 are allowed in view of the Declaration (Paper No. 12) dated October 30, 2001 by Dr. Jones.

The rejection of claims 9-16 as "unpatentable over the Arcadi references (1986) and (1990) of record" is not understood. These claims define the solution which the inventor found to be effective in treating human prostate cancer. The October 30, 2001 Declaration by Dr. Jones makes it clear that "Dr. Arcadi's 1986 and 1990 articles would not cause one of ordinary skill in this work to reasonably expect that Rhodamine-123 would be any more effective in combating human prostate carcinoma than any of many other drugs which have been tested *in vitro* and in laboratory animals with promising results, but which have failed to produce any therapeutic effects in human patients." (See paragraph no. 7 of the Declaration.) Nothing in either of Dr. Arcadi's articles discloses or suggests a solution of Rhodamine-123 in an alcohol and water, which can be used to prolong the life of victims of prostate carcinoma.

Applicant does not understand the statement in the Office action that "Clearly, a showing of the prior pharmaceutical composition is needed." Nothing in either of the Arcadi articles discloses or even suggests the Rhodamine-123 solution defined in claims 9-16 for effective treatment of human prostate carcinoma. If this rejection is repeated, please cite authority for it.

The rejection of claims 20 and 22-24 as "unpatentable over the Bernal et al. reference" is not understood. The animal experiments referred to in the Bernal et al. reference are no more relevant than those described in the Arcadi references, and were disposed of by the Jones Declaration referred to above.

Claims 20-24 were rejected "as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time of the application was filed, had possession of the claimed invention. No basis is seen in the specification and claims as filed to the terms [sic] 'carcinoma'." The accompanying Declaration ("second Declaration") dated July 22, 2002 by Dr. Lawrence W. Jones (attached as Exhibit "A") makes it clear that the subject matter objected to in the Office action is not "new matter". The application as originally filed disclosed how to treat prostate cancer successfully. Prostate cancer is one of many forms of carcinoma, which is any cancer of epithelial origin. That includes cancer of the breast, liver, pancreas, bladder, lung, skin, colon, and the like. Moreover, the application states on page 1 at line 24 that "Rh-123 is selectively toxic for carcinoma cells".

The second Declaration of Dr. Jones also makes it clear that the application describes how to administer Rhodamine-123 safely to patients so the efficacy of the drug for treating all types of carcinoma can be determined by routine experiments, i.e., without undue experimentation.

In view of the explanation set forth in the attached Declaration of Dr. Jones, the objection to the Amendment filed January 8, 2002 as introducing new matter should be withdrawn.

New claim 28 added by this Amendment is similar to allowed claim 1, except that claim 28 is not limited to treatment of prostate cancer. Applicant is entitled to the more generic claim because it has long been the law that "A specification may, within the meaning of 35 U.S.C. § 112, ¶ 1, contain a written description of a broadly claimed invention without describing all species that claim encompasses." *Utter v. Hiraga*, 845 F.2d 993 (Fed. Cir. 1988). Claim 28 should not be rejected because of "overbreadth". "[T]hat word . . . has long ago been discredited as a basis for determining



Application No. 09/383,114

sufficiency of a specification". See *In re Marzocchi*, 439 F.2d 220, 223, 169 U.S.P.Q. 367, 369, 58 C.C.P.A. 1069 (1971), which held that the Patent Office should be concerned with support or non-support of a generic term, not its breadth. In the present case, the treatment of carcinoma is clearly supported by the disclosure and the application.

This application is in condition for allowance, and such action at an early date is requested.

Respectfully submitted,

CHRISTIE, PARKER & HALE, LLP

By R. W. Johnston

R. William Johnston

Reg. No. 17,968

626/795-9900

RWJ/mas

Enclosure: Exhibit A

CLS PAS450049.1-\* - 7/25/02 12:47 PM

## **EXHIBIT A**

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John A. Arcadi  
Application No. : 09/383,114  
Filed : August 25, 1999  
Title : COMPOSITION AND METHOD FOR  
TREATING CARCINOMA (as amended)

Grp./Div. : 1614  
Examiner : J. Goldberg

Docket No. : 35687/RWJ/H29

## DECLARATION

Assistant Commissioner for Patents  
Washington, D.C. 20231

Post Office Box 7068  
Pasadena, CA 91109-7068

Commissioner:

I, Lawrence W. Jones, declare that:

1. I am a licensed physician in the State of California. I am also the Director of Prostate Research Program at Huntington Medical Research Institutes (HMRI), a non-profit medical research organization and the assignee of the above patent application. I have been working in the field of treating prostate cancer for more than 28 years, and have worked as an unpaid volunteer for HMRI since 1974. I am thoroughly familiar with the patent application, and I have no financial interest in it.

2. The patent application discloses the use of rhodamine-123 for treating hormone-refractory prostate cancer, which kills 40,000 men annually in the United States. Before this invention, there was no known life-prolonging treatment for this disease.

3. I am also familiar with the Office action dated 03/11/02, and with an Amendment filed November 6, 2001 (erroneously referred in the Office action as "filed on 08 January 2002"). The Office action contends that the original disclosure of the patent application does not support certain material added by the Amendment, namely, the word "carcinoma" and the term "one of many carcinomas, such as cancer of the breast, liver, pancreas, bladder, lung, skin, colon, and the like." As explained below,

the material objected to in the Office action, is not "new matter". It merely states the meaning of "carcinoma", as it has long been understood by ordinary workers in the field of oncology.

4. The present patent application, and each of its two parent applications, originally disclosed the treatment of carcinoma. For example, the present application describes in detail how to treat prostate cancer with rhodamine-123. It was well known to physicians and other workers in the field of oncology long before the filing date of the present application that prostate cancer is one form of carcinoma, and that carcinoma is any of the various types of malignant tumor derived from epithelial tissue, including cancer of the breast, liver, pancreas, bladder, lung, skin, and colon, as well as other organs of the human body. See Stedman's Medical Dictionary, 24<sup>th</sup> Edition, published 1982, page 223, which is attached as Exhibit "A".

5. The patent application, as originally filed, states on page 1, line 24, that "Rh-123 is selectively toxic for carcinoma cells." Accordingly, the patent application as originally filed makes clear to ordinary workers in this field that rhodamine-123 is useful for treating carcinoma, such as prostate cancer.

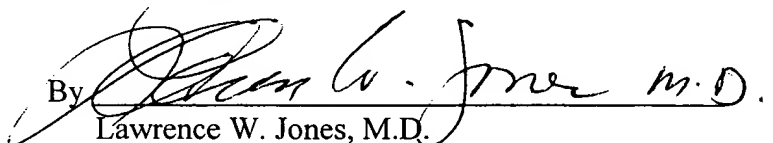
6. The application also makes it clear how to administer rhodamine-123 safely to patients, so that the efficacy of the drug for treating all types of carcinoma can be determined by routine experiments.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date

7/22/2002

By

 m.d.

Lawrence W. Jones, M.D.

RWJ/mas

Attachment : Exhibit A

RWJ PAS449743.1-7/22/02 11:05 AM

## **EXHIBIT A**

ILLUSTRATED  
**Stedman's**  
**MEDICAL**  
**DICTIONARY**  
24TH EDITION



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WILLIAMS & WILKINS  
Baltimore/London

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Baltimore, MD 21202, U.S.A.

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*Made in the United States of America*

English Language Co-editions

Asian 1967, 1972, 1976  
Indian 1967, 1973  
Taiwan 1972, 1978

Translated Editions

Greek 1976  
Indian 1977  
Japanese 1977  
Portuguese 1976  
Spanish (in press)

Library of Congress Cataloging in Publication Data

Stedman, Thomas Lathrop, 1853-1938.  
Stedman's Medical dictionary.

1. Medicine—Dictionaries. I. Title. II. Title: Medical dictionary. [DNLM: 1. Dictionaries, Medical. W 13 S812m]  
R121.S8 1982 610'.3'21 81-3021  
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## carbon

## carbon

223

## carcinoma

'bo-an-jī-og'rā-fī). A form of carbon dioxide is injected

lenzyloxycarbonyl.

See carbonium.

nohemoglobin.

m for enzymes that hydro-

class name for the aldehydic hydroxy alcohols, the name that the most common have formulas that may be  $C_6(H_2O)_6$ ; sucrose, not true hydrates and the dimer. The group includes all molecules, such as the disaccharides, etc.), as well as such as starch, glycosides. The c.'s most typical hydrogen, and oxygen only. Intermediates in tissue contain carbohydrates are c.'s combined

term denoting the excretion in the urine, e.g., glucose, thus including such conditions, galactosuria, lactosuria.

s. carbolyze.

in). 1. See Ziehl's stain. 2.

dd carbolic acid (phenol). *G. ouren*, urine]. The presence in the urine.

ylic acid cross-linked with a base, a poly (acrylic acid) or agent for pharmaceuticals.

nonmetallic tetravalent element, atomic weight 12.01. It has two isotopes (the former, set at 12 for all molecular weights), isotopes of interest,  $^{12}C$  and  $^{13}C$  pure forms, diamond and graphite, coal, coke, and soot; and compounds are found in all forms of its vast number of organic chemistry.

. of *N*-carboxybiotin (biotin form in which c. dioxide is added to form  $\beta$ -methylglutamate and to acetyl-CoA to form l-CoA carboxylase. of a sugar; C-1 of an aldose.

ylene. hydride; carbonic acid gas. solution of c. with an excess of less than 99.0% by volume of solvent. solid c. dioxide; used in the treatment of skin affections.

c. CS<sub>2</sub>; an extremely flammable characteristic ethereal odor, but is seldom used

c., odorless, and poisonous gas. Its action is its toxic action for hemoglobin and cytochrome and blocking oxygen

methane; CCl<sub>4</sub>; a colorless, characteristic ethereal odor resembling that of a cleansing fluid and

as a fire extinguisher, and has been used as an anthelmintic, especially against hookworm.

**carbon-11** ( $^{11}C$ ). A cyclotron-produced, positron-emitting radioisotope of carbon with a half-life of 20 minutes.

**carbon-12** ( $^{12}C$ ). The standard of atomic mass, 98.89 per cent of natural carbon.

**carbon-13** ( $^{13}C$ ). A natural isotope, 1.11 per cent of natural carbon.

**carbon-14** ( $^{14}C$ ). A beta-emitter with a half-life of 5730 years, widely used as a tracer in studying various aspects of metabolism, notably photosynthesis; naturally occurring  $^{14}C$ , arising from cosmic rays, is used to date relics containing natural carbonaceous materials.

**carbonate**. A salt of carbonic acid;  $CO_3^{2-}$ . c. dehydratase (EC 4.2.1.1), c. hydro-lyase; carbonic anhydrase; a zinc-containing enzyme in red blood cells that catalyzes the conversion of carbon dioxide ( $CO_2$ ) entering the blood from the tissues into carbonic acid ( $H_2CO_3$ ). The reverse reaction occurs when the blood reaches the lungs and carbon dioxide is liberated.

c. hydro-lyase, c. dehydratase.

**carbon'ic**. Relating to carbon.

**carbon'ic acid**.  $H_2CO_3$ , formed from  $H_2O$  and  $CO_2$ .

**carbon'ic anhy'drase**. Carbonate dehydratase.

**carbon'ic anhy'dride**. Carbon dioxide (1).

**carbon'ium**. An organic cation in which the positive charge is on a carbon atom. It is now recommended that carbonation be used as the class name and carbenium ( $CH_3^+$ ) be used for specific compound names.

**carbonize**. To char.

**carbonom'eter** [L. *carbo* (carbon-), coal, + G. *metron*, measure]. An obsolete device for determining the proportion of carbon dioxide in the air or expired breath by the precipitation of calcium carbonate from lime water.

**carbonom'etry**. Carbometry; an obsolete method for the determination of the presence and the proportion of carbon dioxide by means of the carbonometer.

**carbonu'ria**. Rarely used term denoting the excretion of carbon dioxide or other carbon compounds in the urine.

**carbonyl**. The characteristic group,  $-CO-$ , of the ketones, aldehydes, and organic acids.

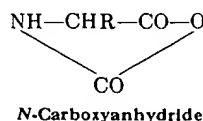
**carborun'dum**. Carbide of silicon;  $SiC$ ; a substance of extreme hardness used for polishing in place of emery.

**carbox'amide**. Aminocarbonyl; a molecular configuration ( $-CONH_2$ ) that, together with the related carboximides (aminocarbonyls) ( $-CONH-$ ), is a constituent of many hypnotics, including barbiturates, hydantoins, and thiazines.

**carboxy-**. Combining form indicating addition of CO or  $CO_2$ .

***N*-carboxyaminoacid anhydrides**. See *N*-carboxyanhydrides.

***N*-carbox'yanhy'drides**. Heterocyclic derivatives of amino acids from which polypeptides may be synthesized.



**carbox'yca'thop'sin**. Dipeptidyl carboxypeptidase.

**carbox'ydis'mutase**. Ribulosebiphosphate carboxylase.

**carbox'yhemoglo'bin (HbCO)**. Carbon monoxide hemoglobin; a fairly stable union of carbon monoxide with hemoglobin. The formation of c. prevents the normal transfer of carbon dioxide and oxygen during the circulation of blood; thus, increasing levels of c. result in various degrees of asphyxiation, including death.

**carbox'yhemoglobine'mia**. The presence of carboxyhemoglobin in the blood.

**carbox'yl**. The characterizing group ( $-COOH$ ) of certain organic acids; e.g.,  $HCOOH$  (formic acid),  $CH_3COOH$  (acetic acid), etc.

**carbox'ylase**. One of several carboxy-lyases, trivially named carboxylases or decarboxylases (EC group 4.1.1), catalyzing the addition of  $CO_2$  to all or part of another molecule to create an additional  $-COOH$  group (e.g., ribulosebiphosphate carboxylase, EC 4.1.1.39).

**carboxyla'tion**. Addition of  $CO_2$  to an organic acceptor, as in photosynthesis, to yield a  $-COOH$  group; catalyzed by carboxylases.

**carbox'yltrans'ferases** (EC group 2.1.3). Transcarboxylases; enzymes transferring carboxyl groups from one compound to another.

**carbox'y'pept'idases**. Hydrolases removing the amino acid at the free carboxyl end of a polypeptide chain.

**carboxypeptidase A** (EC 3.4.17.1). Carboxypolypeptidase; a hydrolase that releases C-terminal amino acids, with exception of arginine, lysine, and proline.

**carboxypeptidase B** (EC 3.4.17.2). Protaminase; a hydrolase that releases C-terminal lysine or arginine preferentially.

**carboxypeptidase C**. Acid carboxypeptidase.

**carboxypeptidase G**.  $\gamma$ -Glutamyl hydrolase.

**carboxypolypeptidase**. Carboxypeptidase A.

***N*-carbox'y'urea**. Allophanic acid.

**carbuncle** (kar'bung-kul) [L. *carbunculus*, dim. of *carbo*, a live coal, a carbuncle. CARB-]. 1. Deep-seated pyogenic infection of several contiguous hair follicles, with formation of connecting sinuses; often preceded or accompanied by fever, malaise, and prostration. 2. Anthrax (1).

**kidney or renal c.**, severe inflammation within the kidney, usually resulting from the coalescence of multiple intrarenal abscesses into a massive intrarenal nest of infection which may open into the renal pelvis or may extend outward and give rise to perinephric abscess.

**carbun'cular**. Relating to a carbuncle.

**carbunculo'sis**. A condition marked by the occurrence of several carbuncles simultaneously or within a short period of time.

**carbure't**. 1. Archaic for carbide. 2. To combine with carbon. 3. To enrich a gas with volatile hydrocarbons, as in a carburetor.

**carbu'tamide**. Aminophenurebutane; 1-butyl-3-sulfamylurea; an oral hypoglycemic agent.

**carbu'terol hydrochloride**. [5-[2-(*tert*-Bu-tylamino)-1-hydroxyethyl]-2-hydroxyphenyl]urea monohydrochloride; a sympathomimetic drug with bronchodilatory activity.

**carcass** (kar'kas) [F. *carcasse*, fr. It. *carcassa*]. 1. The body of a dead animal. 2. In butcher's terminology, refers to animals used for human food; the body after the head, tail, extremities, and viscera have been removed.

**carcin-**. See carcino-.

**carcino-**, **carcin-** [G. *karkinos*, crab, cancer. CARC-]. Combining form relating to cancer.

**carcinogen** (kar'si-no-jen). Any cancer-producing substance. The most potent c.'s, including those isolated from coal tar, are polycyclic aromatic hydrocarbons.

**car'cinogen'esis** [carcino- + G. *genesis*, generation]. The origin or production of cancer, including carcinomas and other malignant neoplasms.

**car'cinogen'ic**. Cancerogenic; causing cancer.

**car'cinolyt'ic** [carcino- + G. *lytikos*, causing a solution]. Destructive to the cells of carcinoma.

## CARCINOMA

**carcinoma** (CA) (kar-si-no'mah) [G. *karkinōma*, fr. *karkinos*, cancer, + suffix *-oma*, tumor]. Any of the various types of malignant neoplasm derived from epithelial tissue in several sites, occurring more frequently in the skin and large intestine in both sexes, the bronchi, stomach, and prostate gland in men, and the breast and cervix in women. C.'s are identified histologically on the basis of invasiveness and the changes that indicate anaplasia, i.e., loss of polarity





ex B

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John A. Arcadi  
Application No. : 09/383,114  
Filed : August 25, 1999  
Title : COMPOSITION AND METHOD FOR  
TREATING PROSTATE CANCER

Grp./Div. : 1614  
Examiner : J. Goldberg

Docket No. : 35687/RWJ/H29

DECLARATION

Assistant Commissioner for Patents  
Washington, D.C. 20231

Post Office Box 7068  
Pasadena, CA 91109-7068

Commissioner:

I, Lawrence W. Jones, declare that:

1. I am a licensed physician in the State of California, and the Director of Prostate Research Program at Huntington Medical Research Institutes (HMRI), the assignee of this patent application.

2. This patent application covers the use of Rhodamine-123 for treating hormone-refractory prostate cancer, which kills 40,000 men annually in the United States. Prior to this invention, there was no known life-prolonging treatment for this disease.

HMRI filed with the U.S. Food and Drug Administration (FDA) an Investigational New Drug Phase I Application, which was approved for the experimental testing of Rhodamine-123 to treat human prostate cancer *in vivo*. Clinical testing to determine a safe dosage level of Rhodamine-123 for humans began under my supervision as Principal Investigator on February 1, 1999. Under Phase I of the approved protocol, 27 volunteer patients are to be treated in nine groups of three each, with each volunteer receiving a single dose of Rhodamine-123.

3. Once the current study has been completed, volunteers will thereafter be treated with multiple doses of Rhodamine-123, starting at a dosage that has been established as non-toxic.

**Application No. 09/383,114**

4. We have completed infusion of 12 volunteers with Rhodamine-123 and have data showing the percentage change of prostate specific antigen (PSA) two weeks after infusion. Prior to treatment, each volunteer was diagnosed as having hormone-refractory prostate cancer (HRPC), which usually leads to death within about two years after the onset of the condition. Each volunteer was treated in accordance with protocol approved by the FDA. The attached graph shows the PSA change for each volunteer two weeks after infusion with Rhodamine-123. To date, no sign of toxicity has been observed in any of the volunteers tested.

5. As indicated on the attached graph, volunteers 1, 2 and 3 were each infused with 0.3mg of Rhodamine-123 per kg of patient body weight (0.3mg/kg). Volunteers 4, 5 and 6 were each infused with a dose of 0.6mg/kg. Volunteers 7, 8 and 9 were each infused with a dose of 1.2mg/kg, and volunteers 10, 11 and 12 each received 2.4mg/kg.

6. As shown in the accompanying graph, volunteers 3, 4, 8 and 12 each experienced a substantial decrease in PSA. The drop exceeded 50% for volunteers 8 and 12. This is important because Kantoff, P.W., et al. reported in the *Journal of Clinical Oncology*, Vol. 17, No. 8 (August), 1999:pp 2506-2513, at page 2509 that median survival time for patients with hormone refractory prostate cancer (HRPC) is significantly extended if a patient has a 50% or greater drop in PSA level after treatment. A copy of the Kantoff, P.W., et al. paper is enclosed.

7. Although the results to date must be considered only preliminary, they are encouraging because of the significant reduction of PSA levels in some of the patients, although each patient received only one dose of a relatively small amount of Rhodamine-123. Moreover, volunteer 12 entered the study with lower limb edema. This had been diagnosed as due to lymphatic obstruction by a tumor in the pelvis of the patient. When seen by the physician one month after treatment with Rhodamine-123 as referred to above, the edema was almost absent, suggesting a possible reduction in tumor mass.

8. We are continuing clinical testing under Phase I of the approved protocol. Once dose-limiting toxicity has been obtained with a single dose of Rhodamine-123, subsequent volunteers will each be treated with multiple doses of Rhodamine-123 at the maximum tolerated dose. That will complete Phase I of the investigation, and should provide further evidence of the efficacy of the treatment.

**Application No. 09/383,114**

9. It is difficult to estimate when clinical testing will be completed, but based on past experience, it is not likely to be done before the end of 2001. The clinical trials and time spent over which they are run depend on the rate at which qualified volunteers can be found and enrolled in the program in accordance with the approved protocol.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

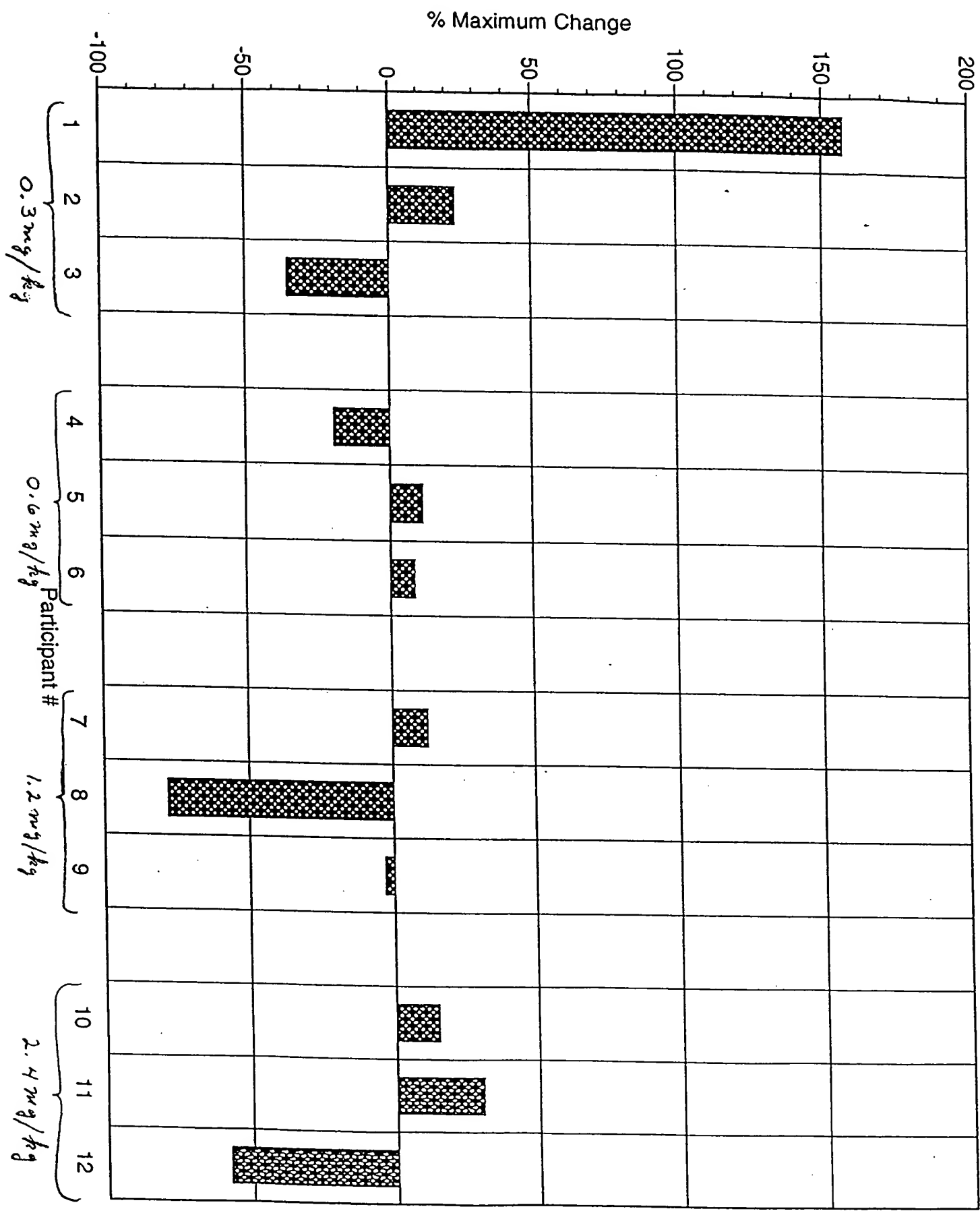
Date 2/23/2001

By 

Lawrence W. Jones, M.D.

Enclosures: Graph  
Kantoff, P.W., et al. publication

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# Hydrocortisone With or Without Mitoxantrone in Men With Hormone-Refractory Prostate Cancer: Results of the Cancer and Leukemia Group B 9182 Study

By Philip W. Kantoff, Susan Halabi, Mark Conaway, Joel Picus, Jeffrey Kirshner, Vera Hars, Donald Trump, Eric P. Winer, and Nicholas J. Vogelzang

**Purpose:** Approximately 40,000 men die each year of hormone-refractory prostate cancer (HRPC). The results of treatment with chemotherapy have been disappointing to date, with no trials demonstrating a benefit with respect to survival duration. Corticosteroids and mitoxantrone each have been shown to be active agents in this disease. The purpose of this study was to demonstrate an advantage of mitoxantrone and hydrocortisone (M+H) over hydrocortisone alone with respect to survival duration.

**Patients and Methods:** Two hundred forty-two patients with HRPC were randomized to receive either M+H or hydrocortisone alone. Patients were monitored for survival, time to disease progression, time to treatment failure, response, and quality-of-life (QOL) parameters.

**Results:** Treatment in both arms was well tolerated. Although there was a delay in time to treatment failure and disease progression in favor of M+H over hydro-

cortisone alone, there was no difference in overall survival (12.3 months for M+H v 12.6 months for hydrocortisone alone). There was an indication that QOL was better with M+H, in particular with respect to pain control.

**Conclusion:** M+H generated more frequent responses and a delay in both time to treatment failure and disease progression compared with hydrocortisone alone. In addition, there was a possible benefit of M+H with respect to pain control over hydrocortisone alone. No improvement in survival was observed. Although M+H could be viewed as a palliative option for patients with HRPC, new drugs and novel strategies are needed to improve survival for this disease.

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PROSTATE CANCER IS the most commonly diagnosed malignancy in men. Although many men are cured with treatment, approximately 40,000 men in the United States die of this disease annually.<sup>1</sup> Androgen withdrawal therapy remains the mainstay of treatment for men with advanced disease. When tumors become refractory to androgen withdrawal therapy, further systemic treatment has been of only modest benefit. Chemotherapy has had an undefined impact on the survival duration and quality of life (QOL) of patients. The few randomized clinical trials using chemother-

apy usually have been underpowered to detect small differences in outcome.<sup>2</sup> Perhaps more importantly, the agents used in such clinical trials have possessed only marginal activity.

Corticosteroids may act in a variety of ways in men with prostate cancer. As a treatment strategy, corticosteroids represent a minimally toxic, low-cost therapy with some activity against prostate cancer and with an apparent beneficial effect on QOL.<sup>3</sup> Mitoxantrone is an anthracenedione that has demonstrated activity in a variety of malignancies, including prostate cancer.<sup>4,5</sup> It is a drug that is well suited for the population of men with advanced prostate cancer because it causes relatively modest toxicity. In 1992, the Cancer and Leukemia Group B (CALGB) approved a trial, CALGB 9182, to compare hydrocortisone alone to hydrocortisone plus mitoxantrone (M+H). The primary end point of this study was survival duration. The results are reported here.

## PATIENTS AND METHODS

### Study Design

Patients were eligible for CALGB 9182 if they had metastatic prostate cancer and had undergone no more than one prior endocrine manipulation. Patients were required to have adequate hepatic, renal, and bone marrow function. Continued use of luteinizing hormone-releasing hormone agonist was required for those who had not undergone an orchiectomy. Antiandrogen withdrawal and subsequent documented disease progression was required before entry soon after the trial was opened. When the trial was activated (June 1992), two

From the Lank Center for Genitourinary Oncology and Breast Oncology Center, Department of Adult Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; Cancer and Leukemia Group B Statistical Center, Duke University Medical Center, Durham, NC; Division of Biostatistics and Epidemiology, University of Virginia Health Sciences Center, Charlottesville, VA; Division of Medical Oncology, Washington University Medical Center, St Louis, MO; Syracuse Hematology-Oncology Community Clinical Oncology Program, Hematology-Oncology Associates of Central New York, Syracuse, NY; Division of Medical Oncology, University of Pittsburgh Medical Center, Pittsburgh, PA; and Section of Hematology Oncology, Department of Medicine, University of Chicago Medical Center, Chicago, IL.

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Address reprint requests to Philip W. Kantoff, MD, Department of Medical Oncology, Dana-Farber Cancer Institute, 44 Binney St, Boston, MA 02115-6084; email philip\_kantoff@dfci.harvard.edu.

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stratification factors were used: performance status (0 to 1 v 2) and disease status (measurable v assessable). After 60 patients were accrued, the eligibility criteria were changed to allow entry of patients who had undergone more than one prior endocrine manipulation, and a third stratification factor was added: the number of prior endocrine manipulations (1 v  $\geq 2$ ). The study was not blinded; patients and physicians knew the treatment assignment. Hydrocortisone was administered orally at a dose of 30 mg in the morning and 10 mg in the evening, and mitoxantrone was administered by intravenous injection at a dose of 14 mg/m<sup>2</sup> every 3 weeks. Dose modifications were mandated for hematopoietic toxicity. Use of growth factors was discouraged. Patients in the hydrocortisone-alone arm were not permitted to cross over to mitoxantrone or doxorubicin. However, alternative chemotherapy was permitted after disease progression in either treatment arm.

From October 1992 to September 1995, 242 patients were registered onto CALGB 9182, with 119 randomized to receive M+H and 123 patients randomized to receive hydrocortisone alone. Four patients, two in each of the treatment arms, never started treatment. Four patients, one in the M+H arm and three in the hydrocortisone-alone arm were ruled ineligible; one patient did not have prostate cancer, one had too low a platelet count, one had too low a hemoglobin level, and one had too high a platelet count. All four of these patients were included in the survival analysis.

The study was designed to detect a 50% increase in survival duration with M+H compared with hydrocortisone alone. Sample size calculations were based on having adequate power (80%) to detect a difference in survival duration under the assumption that the median survival duration in the population of patients who received hydrocortisone alone would be 12 months, compared with 18 months for patients treated with M+H. The expanded eligibility (ie, including patients with more than one prior endocrine manipulation) allowed for patients with potentially poorer prognosis to be entered onto the study, and the sample calculations were amended accordingly but were still based on having 80% power to detect a difference for a hazards ratio of 1.5. Allowing for a 5% ineligibility rate and with a 2-year follow-up period after the accrual period, the target accrual was set at 232 patients. The study closed on September 15, 1995, with a final accrual of 242 patients.

### End Points

The primary end point was survival duration, which was defined as the time between randomization and death. For living patients, the survival time was censored at the time of last follow-up. The secondary end points were time to disease progression, time to treatment failure, best response, measures of QOL, and decrease in serum prostate-specific antigen (PSA). The study required serum PSA determinations every 3 weeks and a bone scan every 2 months for the first 4 months, and then every 3 months thereafter. Other scans were mandated by the presence of measurable disease and were performed every 2 months. Patients were evaluated for best response in one of three categories: measurable disease, assessable disease, or bone-only disease. In all categories, a complete response (CR) was defined as disappearance of all disease by scans and normalization ( $\leq 4$  ng/mL) of serum PSA, both of which needed to be sustained for  $\geq 28$  days. For patients with measurable disease, a partial response (PR) was defined as a  $\geq 50\%$  reduction in bidimensional measurable disease for  $\geq 4$  weeks or a  $\geq 80\%$  reduction in serum PSA for  $\geq 6$  weeks. For patients with assessable and bone-only disease, PR was defined as a more than 80% reduction in serum PSA sustained for  $\geq 6$  weeks. The criteria for decrease in serum PSA were arbitrarily defined in the protocol as  $\geq 50\%$  and  $\geq 80\%$  reduction of serum PSA from baseline at a follow-up examination anytime between 4 and 8 weeks. Because this definition

did not consider the delayed time to maximum serum PSA decrease in a significant proportion of patients, a post hoc analysis was performed in which the maximum serum PSA decrease ( $\geq 50\%$  and  $\geq 80\%$ ) was determined in each arm, including maximum serum PSA decreases achieved beyond 56 days. Stable disease was defined as per National Prostate Cancer Project criteria.

### Monitoring and Statistical Methods

The study was monitored by the CALGB Data Safety Monitoring Board. The Lan and Demets analog of the O'Brien-Fleming sequential boundary was used to maintain an overall alpha significance level of 0.05 while conducting interim analyses of this study.<sup>6</sup> CALGB Data Management Center personnel were responsible for quality assurance of all data submitted by the participating institutions.

Fisher's exact, Pearson's  $\chi^2$ , and the Kruskal-Wallis tests were used to compare treatment arms on demographic and clinical variables. The Kaplan-Meier product-limit estimator was used to estimate the survival duration, time to disease progression, and time to treatment failure in the two arms.<sup>7</sup> The log-rank test was used to compare the treatment arms with respect to survival duration, time to disease progression, and time to treatment failure.<sup>8</sup> The proportional hazards model was used to assess important factors for predicting survival time.<sup>9</sup> The variables included in the model were age (years), race (white v other), treatment arm (M+H v hydrocortisone alone), baseline performance status (1 v 0), baseline alkaline phosphatase level ( $\geq 165$  U/L v  $< 165$  U/L), baseline lactate dehydrogenase level ( $\geq 227$  U/L v  $< 227$  U/L), baseline hemoglobin level ( $\geq 13$  g/dL v  $< 13$  g/dL), weight loss in the previous 6 months (1% to 5% v none and  $\geq 5\%$  v none), measurable disease (yes v no), previous surgery (yes v no), previous radiotherapy (yes v no), prostatectomy (yes v no), orchiectomy (yes v no), and pretreatment serum PSA level ( $\geq 150$  ng/mL v  $< 150$  ng/mL). The serum PSA measurements were transformed on a logarithmic scale. In addition, repeated measures models<sup>10</sup> were used to compare the arms with respect to ln (PSA) profiles and 14 QOL outcomes over time. All tests were performed using a two-sided alpha value of 0.05.

### Treatment Failure

Treatment failure was defined as disease progression, appearance of unacceptable toxicity, or patient refusal to continue therapy. Disease progression was defined as worsening performance status of  $\geq 1$  or the appearance of two or more new lesions on bone scan, or an increase of serum PSA level  $\geq 100\%$  above the pretreatment serum PSA baseline. Time to treatment failure was the time between randomization and any of the aforementioned end points. Time to disease progression was defined as the time between randomization and disease progression or death.

### QOL Assessment

QOL assessments were to be conducted at study entry, at 6 and 12 weeks after study entry, and then at 12-week intervals. A QOL assessment was also planned for the time of treatment failure. The baseline assessment was completed while the patient was in the clinic. Follow-up interviews were conducted by telephone under the direction of the QOL study coordinator.

Five QOL assessments were used. The Functional Living Index-Cancer (FLIC) is a 22-item questionnaire with each item scored from 1 to 7.<sup>11</sup> Subscales of this instrument include physical well-being (12 items), emotional state (five items), and family disruption (two items). The FLIC was used to provide a global assessment of QOL. The Symptom Distress Scale includes 11 items, each of which is scored

from 1 to 5.<sup>12</sup> Specific items include assessments of appetite and fatigue and two assessments of pain (how often and how severe). This scale was used to provide an in-depth evaluation of cancer-related symptoms. The Sexual and Urologic Functioning scale includes seven items taken from the European Organization for the Research and Treatment of Cancer Prostate Cancer Patients' QOL Questionnaire.<sup>13</sup> Three of the items address sexual functioning, and four items address urologic functioning. All seven items are scored from 1 to 4. The Problems in Daily Activities scale consists of eight items scored from 1 to 5.<sup>14</sup> This scale was intended to provide a detailed evaluation of problems in everyday activities. The Impact of Pain on Daily Activities instrument contains seven items that ask the patient to rate, on a scale from 0 to 10, the impact that their pain has on activities such as sleep and normal work.<sup>15</sup>

## RESULTS

Table 1 lists the characteristics of the 242 patients at baseline. The median age of patients on this study was 72 years, 91% of the patients were white, the median length of time since diagnosis was 3.3 years, and median serum PSA level at study entry was 150 ng/mL. More than 90% of patients had bone metastases, and 85% of patients had a CALGB performance status of 0 or 1. There were no significant differences between the two treatment arms with respect to baseline clinical characteristics other than prior treatment with a progestational agent. Hydrocortisone was continued in all patients until disease progression or treatment failure. Its continuation was encouraged in both treatment arms until death. The median number of cycles of mitoxantrone administered was five.

The main objective of this study was to compare the survival duration of hydrocortisone alone versus M+H. Figure 1 shows that there was no difference in survival between the treatment arms (median duration, 12.6 months for hydrocortisone v 12.3 months for M+H; log-rank test = .08, 1 df,  $P = .77$ ). There was a small but statistically significant difference favoring M+H with respect to time to disease progression (Fig 2) and time to treatment failure (data not shown). Treatment failure and disease progression occurred at a median time of 2.3 months after initiation with hydrocortisone alone compared with a median 3.7 months with M+H (log-rank,  $P = .0254$  for treatment failure and  $P = .0218$  for disease progression).

There were 29 patients (31%) in the M+H arm who progressed according to the measurable disease criteria compared with 28 patients (27%) in the hydrocortisone-alone arm. Sixty-six patients (69%) in the M+H arm progressed according to the bone scan compared with 77 (71%) in the hydrocortisone-alone arm. Progression as measured by PSA included 54 patients (57%) in the M+H compared with 48 (46%) in the hydrocortisone-alone arm. Progression according to performance status included 38 patients (39%) in the M+H arm versus 42 (39%) in the hydrocortisone-alone arm.

Table 1. Baseline Characteristics by Treatment Arm

	M + H		Hydrocortisone Only		P
	%	No. of Patients*	%	No. of Patients*	
<b>Demographics</b>					
Age, years					
Median	72	119	72	123	.548
Interquartile range	67-75		65-75		
White race	88	119	93	123	.238
<b>Metastases†</b>					
Bone	91	119	90	123	.892
Lymph node involvement	21	117	17	121	.238
Lung	9	117	9	121	.957
Liver	9	117	16	110	.610
<b>Years since diagnosis</b>					
Median	3.3	111	3.4	110	.402
Interquartile range	1.9-6.3		1.9-5.2		
<b>Laboratory values</b>					
Hemoglobin, g/dL					
Median	11.9	119	12.4	123	.272
Interquartile range	11-13		11-13		
PSA, ng/mL					
Median	150	119	141	123	.884
Interquartile range	52-362		54-416		
Alkaline Phosphatase, IU/L					
Median	167	113	163	116	.992
Interquartile range	105-317		104-369		
Creatinine, mg/dL					
Median	1.1	119	1.1	123	.835
Interquartile range	0.9-1.3		0.9-1.3		
<b>Prior therapy‡</b>					
Surgical Castration	59	116	61	123	.715
Estrogen	8	115	13	121	.246
LHRH analog	47	117	45	121	.713
Progesterone agent	7	115	18	121	.010
Antiandrogen	69	116	75	122	.267
<b>QOL</b>					
Performance status of 0 or 1	85	119	88	122	.328
No analgesic use	35	118	40	120	.884

Abbreviation: LHRH, luteinizing hormone-releasing hormone.

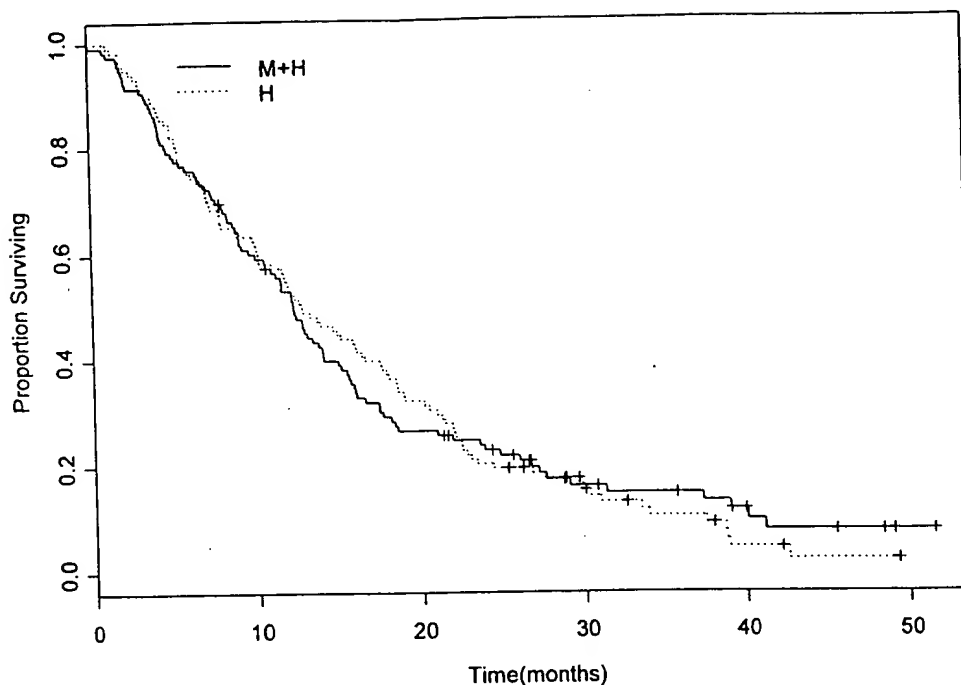
\*Number of patients evaluated on the characteristic.

†Patient may have more than one metastasis.

‡Patient may have more than one type of prior therapy.

Table 2 summarizes the best response by treatment arm as defined in Patients and Methods. This analysis is based on 234 eligible patients who received study treatment. Only 69 patients were considered to have measurable disease. No CRs were observed in either treatment arm. PRs were observed in eight (7%) of 116 patients who received M+H and in five (4%) of 118 who received hydrocortisone alone. There was no significant difference in the CR + PR rates between arms ( $\chi^2$  test = .788; 1 df,  $P = .375$ ). Stable disease was more common with M+H (65 of 116; 56%) versus hydrocortisone alone (50 of 118; 42%). A post hoc analysis showed that 73 (64%) of 116 patients had either a CR, a PR, or stable disease with M+H compared with 55 (47%) of 118 with hydrocortisone alone ( $\chi^2$  test = 6.29, 1 df,  $P = .012$ ).

Fig 1. Overall survival.



There were 1,704 serum PSA measurements, 242 measurements at study entry, and 1,462 after baseline. Of the postbaseline measurements, 849 were in the M+H arm and 613 in the hydrocortisone-alone arm. There were 161 serum PSA measurements after 12 months: 97 in the M+H arm and 64 in the hydrocortisone-alone arm. Table 3 summarizes decrease in serum PSA by treatment arm. The protocol mandated a serum PSA evaluation between 28 and 56 days after initiation of treatment. When the 187 patients who had serum PSA measurements between 28 and 56 days were analyzed, 13 (14%) of 81 patients treated with hydrocortisone alone achieved a  $\geq 50\%$  decrease in serum PSA compared with 18 (19%) of 96 patients treated with M+H. The difference was not statistically significant ( $\chi^2 = .67$ ; 1 df;  $P = .412$ ). Similarly, four patients treated with hydrocortisone alone achieved a  $\geq 80\%$  decrease in serum PSA compared with four patients treated with M+H.

A post hoc analysis showed that 42 (38%) of 112 patients who received M+H achieved a maximum serum PSA decrease of  $\geq 50\%$  compared with 25 (22%) of 116 patients who received hydrocortisone alone ( $\chi^2 = .985$ ; 1 df;  $P = .008$ ). A maximum serum PSA decrease of  $\geq 80\%$  was achieved in 22 (20%) of 112 patients who received M+H compared with 11 (9.0%) of 116 patients who received hydrocortisone alone ( $\chi^2 = 4.752$ ; 1 df;  $P = .029$ ; Table 3). The discrepancy between the response rates determined before as opposed to after 56 days reflects the fact that most patients who had a decrease in serum PSA level to  $\geq 50\%$  or more than 80% did so beyond 56 days after initiation of treatment. Survival by maximum postbaseline serum PSA

decrease is shown in Figure 3. Patients who achieved a  $\geq 50\%$  or a  $\geq 80\%$  decline from baseline in both cases had a median survival duration of 20.5 months, which was 10.3 months longer than those who did not (log-rank,  $P < .001$ ).

Table 4 shows the pretreatment factors that were significant for prediction of survival duration. These factors included baseline lactate dehydrogenase level ( $\geq 227$  U/L v  $< 227$  U/L; hazards ratio = 1.5), baseline hemoglobin level ( $> 13$  g/dL v  $< 13$  g/dL; hazards ratio = 0.7), baseline alkaline phosphatase level ( $\geq 165$  U/L v  $< 165$  U/L; hazards ratio = 1.7), and baseline serum PSA level ( $\geq 150$  ng/mL v  $< 150$  ng/mL; hazards ratio = 1.4). Although performance status and weight loss were significant predictors in univariate analysis, they were not significant predictors in multivariate analysis. Age, race, treatment arm, measurable disease, previous surgery, previous radiotherapy, previous surgery, previous orchiectomy, and number of prior endocrine manipulations all proved insignificant.

Of the 234 eligible patients who started treatment, 196 (84%) completed at least one of the five QOL instruments at baseline, and 183 patients (78%) completed at least one instrument after baseline. A total of 155 patients (66%) were assessed at baseline and at least one follow-up examination. Of the 51 patients who did not have a postbaseline QOL assessment, five died and two had withdrawn consent before the first scheduled QOL assessment at 6 weeks. When patients who did not fill out at least one follow-up questionnaire were compared with those who did, there were significant differences in baseline characteristics. Those who did not complete a follow-up questionnaire had, on average,



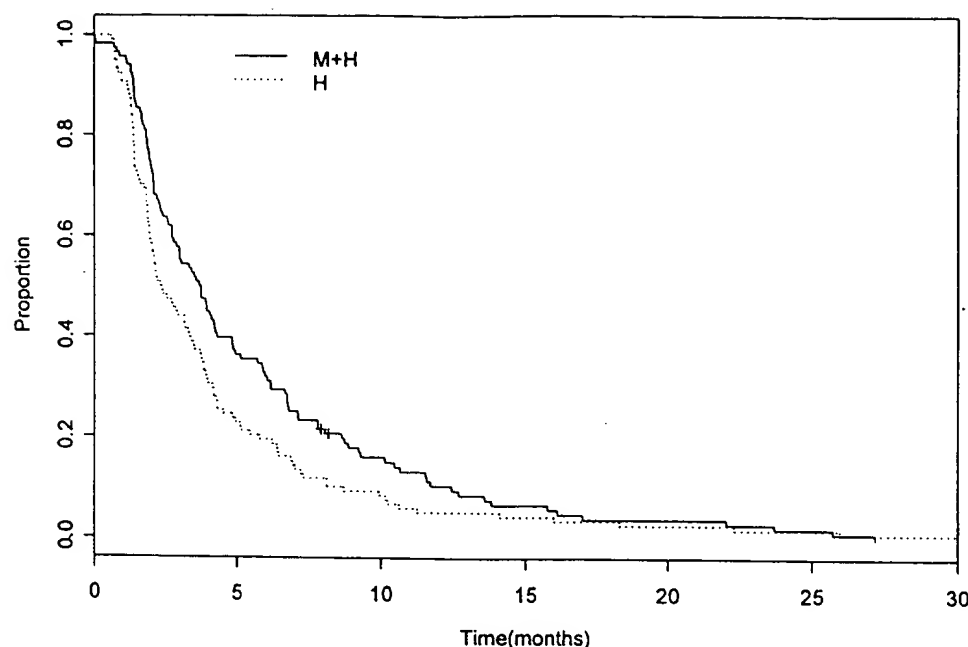


Fig 2. Time to progression.

a poorer performance status and lower QOL scores (as measured by the FLIC). In a multivariate regression analysis, QOL was analyzed in patients who completed a baseline assessment and at least one follow-up assessment. Table 5 summarizes the results of the QOL measures based on estimates of the mean postbaseline average score between treatment groups, adjusting for stratification factors and the baseline score. There were no statistically significant differences between the two arms in a variety of QOL measures, including global QOL (as measured by the total FLIC score), sexual and urologic function, problems of daily activity, and the summary score of the impact of pain scale. However, there was an indication of better QOL in the M+H arm as measured by favorable responses to individual questions and subscales. The differences in the FLIC emotional state subscale ( $P = .04$ ), FLIC family disruption subscale ( $P = .02$ ), and two pain items from the symptom distress scale (how often [ $P = .06$ ] and how severe [ $P = .03$ ]) all favored

the M+H arm. However, the symptom distress scale total and the sexual and urologic function total favored the hydrocortisone-alone arm.

There were no reported treatment-related deaths. The most commonly reported grade 3 and 4 toxicities were hematopoietic toxicity in approximately 70% of patients in the M+H arm (Table 6). The difference between the two arms with regard to hematopoietic toxicity was statistically significant. Cardiac toxicity is a concern with mitoxantrone. We found the rate of grade 3 and 4 cardiac dysfunction to be higher (5%) with M+H than with hydrocortisone alone (0%), but this was not statistically significant. No deaths were attributed to M+H-induced cardiac toxicity.

## DISCUSSION

The results of this study indicate that M+H is more active than hydrocortisone alone, as demonstrated by more frequent decreases in serum PSA levels and a longer time to

Table 2. Greater Than 50% and 80% Reduction in PSA from Baseline at 28 to 56 Days by Arm

PSA Response (%)	Treatment Arm					
	M + H		Hydrocortisone Alone		Total	
	No.	%	No.	%	No.	%
< 50	78	81.3	78	85.7	156	83.4
≥ 50*	18	18.7	13	14.3	31	16.6
≥ 80*	4	4.2	4	4.3	8	4.3

\*The rows ≥ 50% and ≥ 80% are not mutually exclusive; therefore, the number of patients with a PSA response ≥ 50% includes those whose response was ≥ 80%.

Table 3. Greater Than 50% and 80% Reduction in PSA From Baseline by Arm

PSA Response (%)	Treatment Arm					
	M + H		Hydrocortisone Alone		Total	
	No.	%	No.	%	No.	%
< 50	70	62.5	91	78.5	161	70.6
≥ 50*	42	37.5	25	21.5	67	29.4
≥ 80*	22	19.6	11	9.5	33	14.5

\*The rows > 50% and ≥ 80% are not mutually exclusive; therefore, the number of patients with a PSA response > 50% includes those whose response was ≥ 80%.

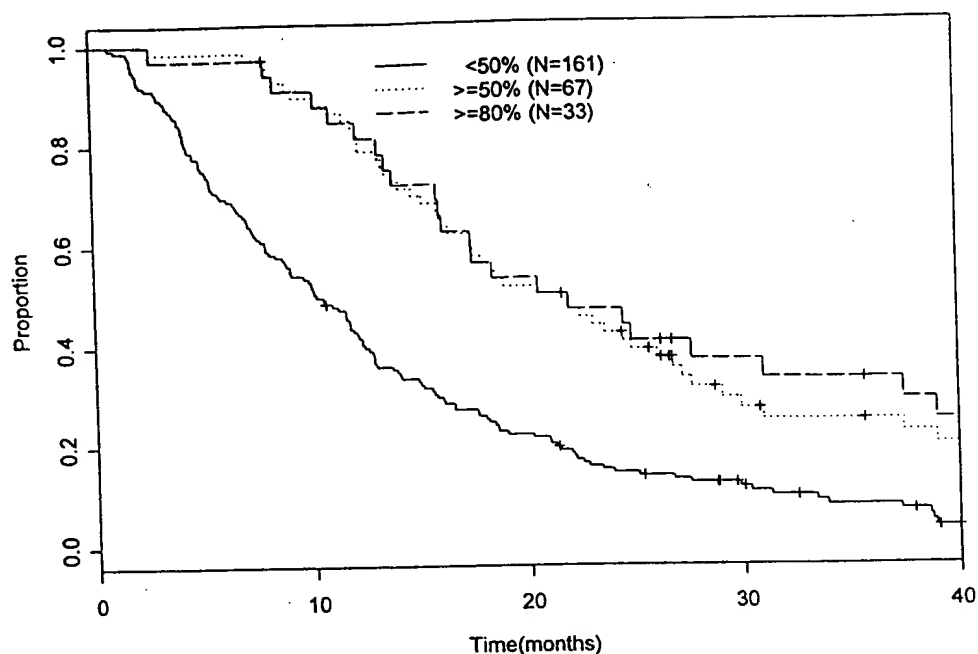


Fig 3. Survival by PSA reduction (n = 228). PSA reduction groups are not mutually exclusive.

treatment failure. However, this difference did not result in a significant improvement in survival, the primary end point of this study. The power calculations for this study were based on an anticipated 12-month survival for the hydrocortisone-alone arm and an expectation that M+H might improve survival by 50%. The estimate of 12-month median survival was correct. Although the 50% improvement in survival might be viewed as an overly optimistic expectation, the decision by the investigators was that a clinically meaningful change in survival for this population would be a 50% improvement in survival. Sensitivity analysis indicated that it is unlikely that a small (> 10%) improvement in survival was missed (data not shown). We believe the primary reason for this is that mitoxantrone lacks sufficient activity in HRPc to translate into a survival benefit. An additional explanation may be that the use of other treatments after treatment failure conceivably could have muted

any survival impact of mitoxantrone. This study did not address the potential utility of mitoxantrone in patients with earlier disease, nor did it address the possibility that other drugs or combination chemotherapy with or without mitoxantrone might improve survival in patients with HRPc.

The results of this study support the use of M+H as a palliative combination in HRPc. Tannock et al<sup>5</sup> demonstrated the added benefit of mitoxantrone plus prednisone over prednisone alone in patients with symptomatic HRPc. In their study, the effect of treatment on pain, analgesic use, and QOL were the primary end points, whereas in our study,

Table 4. Estimates of the Prognostic Factors of Survival From the Proportional Hazards Model

Variable	Hazards Ratio	95% Confidence Limits	P
Alkaline phosphatase $\geq 165$ U/L v < 165 U/L	1.70	1.3, 2.3	< .001
Lactate dehydrogenase $\geq 227$ U/L v < 227 U/L	1.50	1.2, 2.0	.003
Hemoglobin > 13 g/dL v < 13 g/dL	0.70	0.5, 0.9	.021
Baseline PSA $\geq 150$ ng/mL v < 150 ng/mL	1.40	1.1, 1.8	.045
Treatment arm, M + H v hydrocortisone alone	1.0	0.8, 1.3	.976

Table 5. Estimated Treatment Effects, Adjusting for Baseline Score and Stratification Factors

QOL Outcome	Estimated Difference*	SE	P
FUC: total	-4.34	2.74	.12
Symptom distress: total	0.05	0.92	.96
Sexual and urological function: total	0.08	0.57	.89
Problems in daily life: total	-1.25	0.97	.20
Impact of pain: total	-1.87	2.12	.38
FUC: physical well-being	-1.90	1.79	.29
FUC: emotional state	-1.42	0.69	.04
FUC: family disruption	-0.93	0.39	.02
FUC item: pain from cancer	0.35	0.31	.26
FUC item: pain interferes	-0.18	0.22	.43
Symptom distress item: pain, how often	-0.30	0.15	.06
Symptom distress item: pain, how severe	-0.28	0.13	.03
Symptom distress item: appetite	0.08	0.14	.59
Symptom distress item: fatigue	-0.06	0.14	.68

\*Coefficient of indicator for treatment effect: 1 for M + H, 0 for hydrocortisone only. Negative values indicate better QOL with M + H; positive values are in favor of the hydrocortisone-only group.

Table 6. Percentage of Patients with Grade 3 and Greater for Specific Toxicities

Toxicity	Treatment Arm				P†
	M + H		Hydrocortisone Only		
	%*	No.†	%	No.	
WBC	59	112	1	113	< .001
Platelets	6	112	0	112	< .01
Granulocytes/bands	63	112	1	113	< .001
Lymphocytes	70	110	15	111	< .001

\*Percentage of patients within treatment arm with grade 3 or 4 toxicity.

†Number of patients within treatment arm evaluated for the toxicity.

‡Fisher's exact test.

survival was the primary end point. The present study did not demonstrate as robust an improvement of QOL in the M+H arm, although there were elements similar to the study by Tannock et al that favored the M+H arm, specifically, the frequency and severity of pain. Three potential explanations for the difference between our results and the results of Tannock et al are that more than one third of patients in our study at baseline either had no pain or pain that was not sufficient to warrant pain medication, whereas in the study by Tannock et al, pain was a prerequisite. Second, there was a high (34%) dropout rate with regard to assessment of QOL parameters. Both of these factors may have compromised the power to measure a difference in QOL between treatment arms. Finally, the instrument used for measuring QOL in the study by Tannock et al may have been more sensitive than the instruments used in our study. Nonetheless, the results of our study generally support a benefit with respect to pain control of M+H over hydrocortisone alone.

The present study represents one of the largest randomized chemotherapy trial in which an assessment of prognostic factors could be made. The results support the observa-

tions of others that baseline lactate dehydrogenase, alkaline phosphatase, hemoglobin, and PSA levels at baseline are predictive of survival outcome.<sup>16-25</sup>

The results of the analysis of PSA decreases is intriguing. First, maximum PSA decreases were achieved slowly, usually after 2 months of therapy. The difference in the two treatment arms with regard to frequency of decline could only be appreciated after this interval of time. Second, a  $\geq 50\%$  decrease in PSA was associated with a longer survival, supporting the observations of other studies that this may be a clinically meaningful end point in patients with HRPc.<sup>21-23</sup> And finally, no difference in survival was observed in patients who achieved a  $\geq 80\%$  decrease over those who achieved a  $\geq 50\%$  decrease, suggesting that this more rigorous criterion of activity adds no further clinical information than that which is demonstrated by a  $\geq 50\%$  decrease.

Finally, the toxicity observed in this study was very modest, particularly considering the age of the patients. Although there was a high frequency of grade 3 or 4 hematopoietic toxicity in the M+H arm, this did not result in undue morbidity. Neutropenic fevers were unusual, even with the stipulation that growth factor support was discouraged. Given the potential cardiac toxicity of mitoxantrone, it is encouraging that cardiac dysfunction was rarely reported, although exclusively, in the M+H arm. The probable reasons for this are that cardiac function was monitored closely with serial studies and that few patients received large cumulative doses of mitoxantrone.

In conclusion, M+H is beneficial to a proportion of patients with HRPc, and this combination may be used as a control arm in future phase III trials. However, new drugs and combination regimens, as well as novel therapeutic strategies, are needed.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John A. Arcadi  
 Application No. : 09/383,114  
 Filed : August 25, 1999  
 Title : COMPOSITION AND METHOD FOR  
 TREATING CARCINOMA (as amended)



Grp./Div. : 1614  
 Examiner : J. Goldberg

Docket No. : 35687/RWJ/H29

DECLARATION

Assistant Commissioner for Patents  
 Washington, D.C. 20231

Post Office Box 7068  
 Pasadena, CA 91109-7068

Commissioner:

I, Lawrence W. Jones, declare that:

1. I am a licensed physician in the State of California, and the Director of Prostate Research Program at Huntington Medical Research Institutes (HMRI), a non-profit medical research organization and the assignee of the above patent application. I have been working in the field of treating prostate cancer for more than 28 years, and have worked as an unpaid volunteer for HMRI since 1974. I am thoroughly familiar with the patent application, and I have no financial interest in it.

2. The patent application covers the use of rhodamine-123 for treating hormone-refractory prostate cancer, which kills 40,000 men annually in the United States. Before this invention, there was no known life-prolonging treatment for this disease.

3. I am also familiar with the Office actions dated 10/23/00 and 07/13/01, which reject claims 1-27 in this application as unpatentable over the two Arcadi references of 1986 and 1990.

4. HMRI filed with the U.S. Food and Drug Administration (FDA) an Investigational New Drug Phase I Application, which was approved for the experimental testing of rhodamine-123 to treat human prostate cancer *in vivo*. Clinical testing to determine a safe dosage level of rhodamine-123 for humans began under my supervision as Principal Investigator on February 1, 1999. Under Phase I of

**Application No. 09/383,114**

the approved protocol, 27 volunteer patients are to be treated in nine groups of three each, with each volunteer receiving a single dose of rhodamine-123.

5. As stated in my previous Declaration dated 2/23/2001 in this application, clinical testing done under my direction showed a substantial decrease in prostate specific antigen (PSA) for 4 out of 12 patients undergoing clinical tests under my supervision. For 2 of those patients, the decrease in PSA exceeded 50%, which is strong evidence that median survival time for such patients may be significantly extended.

6. Over the past 50 years, hundreds of drugs tested *in vitro* and in laboratory animals have shown potential as antitumor agents, but subsequently failed in clinical tests, or never reached that stage. For example, see U.S. Patent 5,360,803 (filed November 6, 1992, and assigned to Dan Farber Cancer Institute and Fuji Photo Film Co., Ltd.), which discloses at least 348 antitumor agents for treating prostate cancer in humans. I review regularly publications and reports dealing with agents for treating cancer, and as far as I am aware, none of those disclosed by that patent have been accepted by the medical profession as a treatment for prolonging life of patients afflicted with hormone-refractory prostate cancer.

7. Contrary to the statement in the 07/13/01 Office action on page 2, Dr. Arcadi's 1986 and 1990 articles would not cause one of ordinary skill in this work to reasonably expect that rhodamine-123 would be any more effective in combating human prostate carcinoma than any of many other drugs which have been tested *in vitro* and in laboratory animals with promising results, but which have failed to produce any therapeutic effects in human patients. Nothing in either of Dr. Arcadi's articles discloses that life can be prolonged by treating victims of prostate cancer with rhodamine-123.

8. An important problem in the management of prostate cancer is the heterogeneity of the disease. Unequivocal evidence from animal studies, from the growth of human prostate cancer in tissue culture, in the xenograft system, and from human biopsy material shows that many different types of tumor cells exist within prostate cancer. Accordingly, even though a drug may be demonstrated to be effective in laboratory *in vitro* and animal experiments, that does not justify a "reasonable expectation" that it will be effective in treating human prostate cancer. This is well recognized by skilled workers in this field. For example, attached to this Declaration as Exhibit A is a paper by the inventor and others (including me) entitled "Studies of Rhodamine-123: Effect on Rat Prostate Cancer and Human Prostate

Cancer Cells In Vitro,” presented in the *Journal of Surgical Oncology* 59:86-93 (1995), which describes some experimental work providing some basis for this patent application. Under “Editorial Comments” at the end of the paper, Dr. T. Vincent Shankey, with the Departments of Urology and Pathology at Loyola University Medical Center in Maywood, Illinois, rejects the work described in the paper as supporting “the thesis that Rh-123 may be an effective agent for the treatment of metastatic hormone-refractory prostate cancer” because it “is a connection that has too often failed in the past.” Dr. Shankey’s criticism cites other authorities who have pointed out that “the local environment of solid malignancies *in situ* has a profound impact on the responsiveness or nonresponsiveness of cancers where they really count to a patient in his or her body.”

9. Further evidence of skepticism about rhodamine-123 long after Dr. Arcadi’s 1986 and 1990 articles appear in an article entitled “Synthesis and Evaluation of Novel Rhodacyanine Dyes That Exhibit Anti-Tumor Activity” by Kawakami et al., published in the *Journal of Medical Chemistry* in 1997 at 40, 3151-3160 (copy attached as Exhibit B). On page 1 of that article, the authors, referring to various organic compounds, including rhodamine-123, which have been explored as potential antitumor drugs, state that: “In spite of high potential as antitumor agents, none of them have met the criteria for clinical development, such as water solubility, stability, toxicity, and pharmacokinetics”.

10. At least as early as 1982, rhodamine-123 was known to reduce the clonal growth of carcinoma cells *in vitro*. For example, see attached Exhibit C, an article entitled “Rhodamine-123 Selectively Reduces Clonal Growth of Carcinoma Cells *In Vitro*”, published in *Science*, Vol. 218, pp. 1117 & 1118, (10 December 1982). Dr. Arcadi’s work was published in 1986 and 1990. Even so, there was still substantial professional skepticism about the efficacy of rhodamine-123 for treating human prostate cancer. For example, as late as 1997 (see Exhibit B referred to above in paragraph 9), workers at Fuji Photo Film Co., Ltd. and Harvard Medical School dismissed rhodamine-123 as failing to meet the necessary criteria for clinical development.

11. Based on my experience and the skepticism of colleagues with respect to the possible efficacy of rhodamine-123 for treating prostate cancer, the work published by Dr. Arcadi in 1986 and 1990 does not provide a reasonable expectation that rhodamine-123 would be any more effective for combating human prostate cancer than any of many other agents which showed promising laboratory results, and failed to be therapeutic. The drug industry and the medical profession have spent millions of dollars and thousands of research hours seeking an effective therapy for prostate cancer. By any

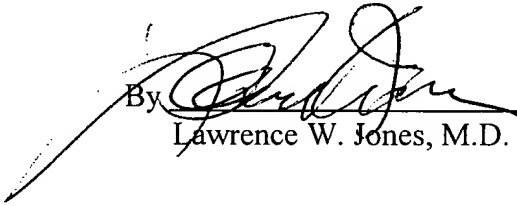
**Application No. 09/383,114**

objective standard, if Dr. Arcadi's 1986 and 1990 articles had actually created a reasonable expectation that treatment with rhodamine-123 would prolong the life of prostate cancer victims, the compound would have been put to wide use instead of being dismissed as clinically inadequate by other workers in that field.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date 10/30/01

By

  
Lawrence W. Jones, M.D.

RWJ/mas

Attachments: Exhibits A-C

CLS PAS390616.1-10/30/01 10:37 AM



## **EXHIBIT A**

## Studies of Rhodamine-123: Effect on Rat Prostate Cancer and Human Prostate Cancer Cells In Vitro

JOHN A. ARCADI, MD, K. SHANKAR NARAYAN, PhD, GEZA TECHY, PhD, CHUEN-PEI NG, BS,  
RAMEZ M.G. SAROUFEEM, MD, AND LAWRENCE W. JONES, MD  
*From the Huntington Medical Research Institutes, Pasadena, California*

The effect of the lipophilic, cationic dye, Rhodamine-123 (Rh-123), on prostate cancer in rats, and on three tumor cell lines in vitro is reported here. The general toxicity of Rh-123 in mice has been found to be minimal. Lobund-Wistar (L-W) rats with the autochthonous prostate cancer of Polard were treated for six doses with Rh-123 at a dose of 15 mg/kg subcutaneously every other day. Microscopic examination of the tumors revealed cellular and acinar destruction. The effectiveness of Rh-123 as a cytotoxic agent was tested by clonogenic and viability assays in vitro with three human prostate cancer cell lines. Severe (60-95%) growth inhibition was observed following Rh-123 exposure for 2-5 days at doses as low as 1.6 µg/ml in all three prostate cancer cell lines. © 1995 Wiley-Liss, Inc.

**KEY WORDS:** mitochondrial toxin, autochthonous rat prostate tumor, chemotherapy, prostate cancer, Rhodamine-123

### INTRODUCTION

Metastatic hormone refractory prostate cancer has responded poorly to chemotherapy because of its slow rate of replication [1]. New agents for treatment of this disease are needed that exert their effect independent of the rate of cell division or of their ability to interfere with DNA or RNA metabolism. We propose Rhodamine-123 (Rh-123) as one such agent. Rh-123, localizing in the mitochondria of living cells [2], is selectively toxic for carcinoma cells because of a difference in the plasma membrane potential of normal and malignant cells together with the positive charge on this lipophilic molecule [3]. In 1986, Arcadi [4] reported the effect of this agent on the transplantable rat prostate tumor R3327-H (Dunning). Rh-123 was administered subcutaneously every other day at a dosage of 15 mg/kg body weight for 52 days. There was significant destructive alteration of the acinar cells with disruption of the cells from the basement membrane, destruction of the cytoplasm, as well as vacuolization and change in fibroblast shape and density.

The highly malignant, androgen-independent transplantable tumor designated P-A III, was reported in 1990 to be highly sensitive to Rh-123 [5]. Rh-123 treatment of the tumor resulted in significant destruction of tumor cells, with no toxicity noted in normal cells. Injection of

tumor remnants into untreated susceptible Lobund-Wistar (L-W) rats produced no tumor growth. An additional rat prostate study is presented in this paper which examines the effect of Rh-123 on the autochthonous rat prostate adenocarcinoma produced in L-W rats by the injection of N-methyl-N-nitrosourea (MNU) and testosterone propionate (TP) [6].

In Arcadi's initial studies [4,5] on rats dimethylsulfoxide (DMSO) was used as a solvent for Rh-123. Since DMSO was not satisfactory for intravenous use in humans, an alcohol-glucose solution was devised. This paper includes a study of the toxicity of these two solvents and Rh-123 in mice. Further studies presented in this paper are those relating to the effect of Rh-123 on various human prostate cancer cell lines. These include the effect of Rh-123 on clonogenicity (plating efficiency), Rh-123 uptake and retention, and Rh-123-induced cytotoxicity.

### MATERIALS AND METHODS

Rh-123, laser grade,  $C_{21}H_{17}ClN_2O_3$  with a molecular weight of 380.83 was purchased from the Eastman Kodak Company (Rochester, NY).

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Address reprint requests to John A. Arcadi, MD, Huntington Medical Research Institutes, 99 N. El Molino Ave., Pasadena, CA 91101.



Fig. 1 (legend on following page).

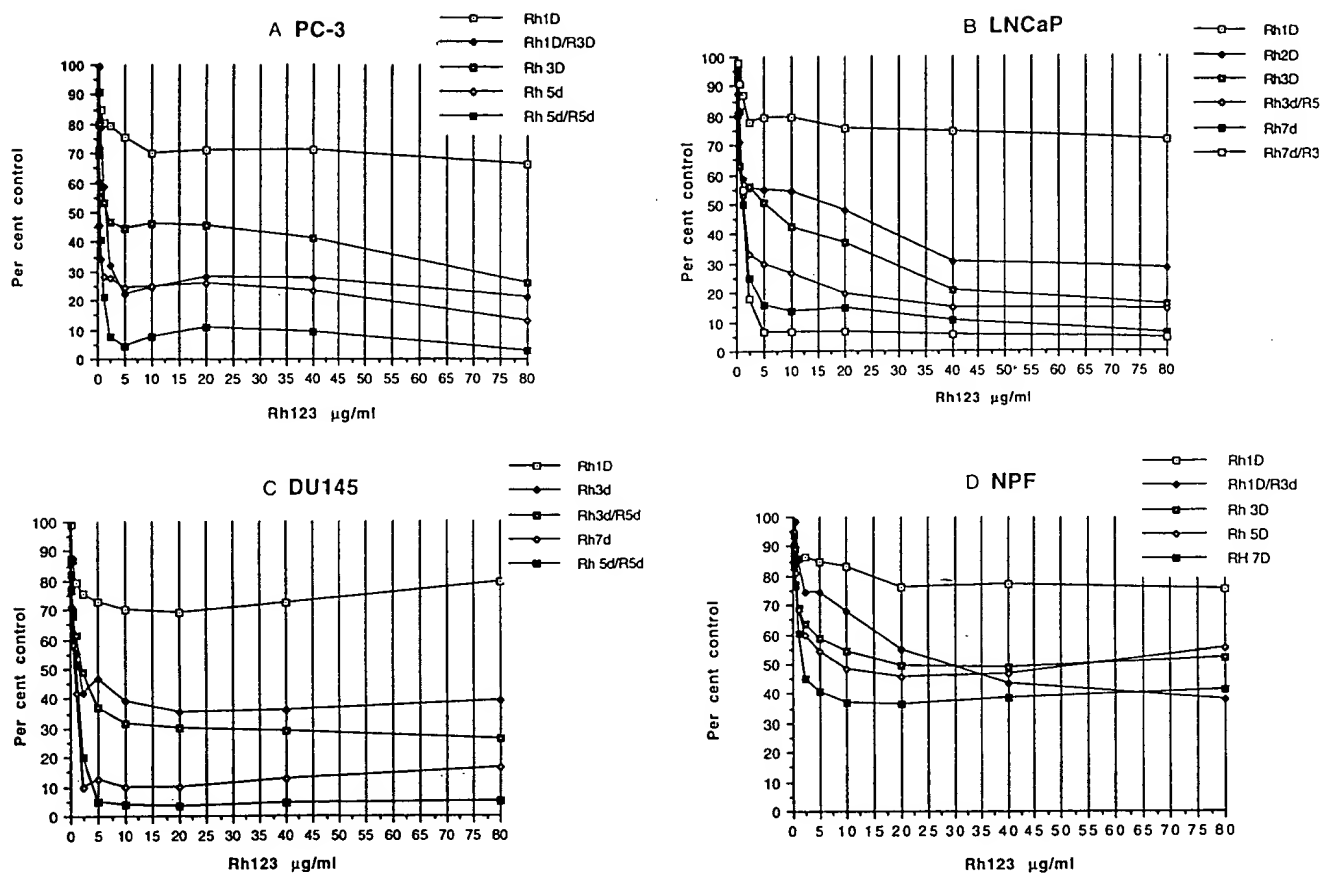


Fig. 2. **A:** Effect of Rh-123 exposure over a 0–80  $\mu\text{g/ml}$  concentration range in PC-3 prostate cancer cells. Data shown as per cent viable cells (expressed relative to the viable cells in control, untreated sister wells) present following exposure to different Rh-123 concentrations for 1, 2, 3, 5, or 7 days. Values shown for each Rh-123 concentration are averages of 8 or 16 duplicate wells. Viability of cells were determined as described in Materials and Methods. *Abbreviations:* Rh1d, Rh2d, Rh3d, Rh7d—cells exposed to Rh-123 continuously for 1, 2, 3 or 7 days. *Rh 1d/R3D*—cells exposed to Rh-123 for 1 day plus recovery in normal growth medium for 3 days before cell viability assessment. *Rh 3d/R5*—Rh-123 exposure 3 days plus recovery for 5 days—cell viability assessment. *Rh7d/R3*—Rh-123 exposure 7 days plus 3 days recovery

period in normal growth medium before cell viability assessment. **B:** Effect of Rh-123 exposure over a 0–80  $\mu\text{g/ml}$  concentration range in LNCaP prostate cancer cells. Data from same experiment as for A. See A for other details and abbreviations. **C:** Effect of Rh-123 exposure over a 0–80  $\mu\text{g/ml}$  concentration range in DU145 prostate cancer cells. Data from same experiment as for A. See A for other details and abbreviations. **D:** Effect of Rh-123 exposure over a 0–80  $\mu\text{g/ml}$  concentration range in NPF non-tumorigenic, diploid prostate cells. *Note:* growth inhibition due to Rh-123 treatment is much less in comparison to that observed with tumorigenic cells (A, B, C). Data from same experiment as for A. See A for other details and abbreviations.

### Rat Prostate Adenocarcinoma

This study was designed to determine the effectiveness of Rh-123 on induced autochthonous rat prostate adenocarcinoma that developed within the prostate gland and seminal vesicles of L-W rats. Thirteen L-W rats were inoculated intravenously with acidified MNU (30 mg/kg

body weight [BW]). Following the single inoculation of MNU, the rats were implanted subcutaneously with TP (50 mg) sealed in a silastic tube. Three implants of TP were administered, each at intervals of 2 months. After a latent period of 4–6 months, small palpable tumors were detected in the abdomen. They were then administered Rh-123 (15 mg/kg BW) subcutaneously every other day for six doses. The Rh-123 was dissolved in a 5% ethanol-5% glucose solution at a concentration of 5 mg/ml. The rats were sacrificed 1 week after the last dose of Rh-123 and their tissues were fixed in 10% formalin.

### Toxicity Studies

The toxicity of two solvents for Rh-123, DMSO and alcohol-glucose, was studied in 60-day-old Swiss-Webster mice (Simonsen Laboratories, Inc., Gilroy, CA). For

Fig. 1. **a:** Untreated autochthonous rat prostate complex adenocarcinoma (ARPCA). Note irregular nuclei with prominent nucleoli; cytoplasm is plentiful and well-defined. **b:** ARPCA treated with Rh-123, 15 mg/kg BW every other day for six doses. Cytoplasm is greatly decreased in volume and the nuclei are smaller and less distinct. Cyst formation is noted in both acini (arrows). **c:** ARPCA treated as in b. Large cyst of cytoplasm between two nuclei is shown. **d:** ARPCA treated as in b. Note smudging and loss staining of cytoplasm. Nuclear detail is lost. Hematoxylin and eosin stained sections. Original magnification  $\times 400$ .

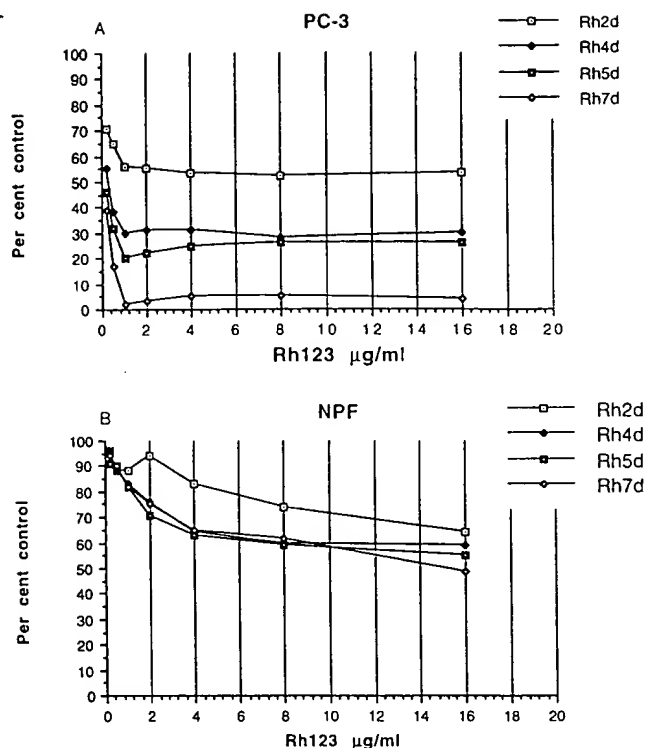


Fig. 3. A: Growth inhibition in PC-3 prostate cancer cells due to Rh-123 exposure at concentrations of 0–16 µg/ml for 1–7 days. This graph better illustrates the maximum cytotoxic effects noted at low Rh-123 concentrations. See Figure 2A for other details and abbreviations. Data shown are from a different experiment than that shown in Figures 2A–2D. B: Growth inhibition in NPF non-tumorigenic prostate fibroblasts due to Rh-123 exposure at concentrations of 0–16 µg/ml for 1–7 days. Compare with A. Data from same as experiment as for A. See Figure 2A for other details and abbreviations.

each solvent group there were six groups of five mice each with the dose per group 2.0 mg/kg, 7.5 mg/kg, and 20 mg/kg. The solvents were at a concentration of 50% for the DMSO, and 5% alcohol in 5% glucose, and the concentration of Rh-123 was 5 mg/ml. The appropriate controls were utilized. Mice were injected subcutaneously every other day for 2 weeks.

### Studies In Vitro

Assays of Rh-123 toxicity were done with three human prostate cancer cell lines, PC-3 [7], DU 145 [8], and LNCaP [9], and a nontumorigenic diploid prostate fibroblast cell strain (NPF-209) derived in our laboratories from a normal adult prostate. The NPF cells were used as controls, for comparison. The cells were maintained in disposable plastic culture vessels in a 1:1 mix of Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12, Sigma Chemical Co, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; HyClone Labs, Inc., Logan, UT). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), DMSO, and other chemicals were obtained from Sigma.

Rh-123 was dissolved in high purity water (Milli Q, Millipore Corp., Bedford, MA) at 2 mg/ml and sterilized by 0.2 µm filtration before use for cell cultures. Two different *in vitro* assays were utilized to assess cell viability following Rh-123 treatment: i) colony formation by a clonal assay procedure [10] and ii) viability of cells determined by the MTT assay utilizing previously described techniques [11].

### Clonogenic Potential of Treated Cells

Clonogenic potential of treated cells was determined on 24-hour old cells seeded in 60 mm disposable dishes ( $1-2 \times 10^2$  cells/dish). The cells were exposed to Rh-123 for 24, 48, or 72 hours in triplicate sets at final concentrations of 1–50 µg/ml. They were then washed, and reincubated with Rh-123-free culture medium for 10–14 days before fixation, staining, and counting of colonies consisting of eight or more cells. Data are reported relative to the number of colonies observed in control (untreated) cultures carried in parallel and represent results confirmed by repeat experiments.

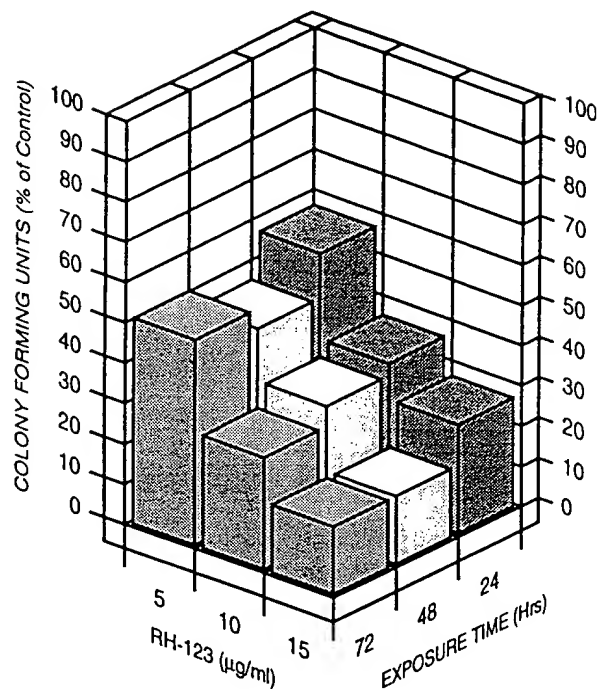
### Cytotoxicity Due to Rh-123

Cytotoxicity due to Rh-123 treatment was determined with cells grown in 96-well disposable microtiter plates. Cells were seeded at  $2.5-4 \times 10^3$  cells per well and allowed to grow in normal culture medium for 2–3 days to obtain cells in exponential growth phase. The cells were then exposed to various Rh-123 concentrations (in sets of eight wells per concentration) spanning the range of 0–80 µg/ml by adding appropriate amounts of sterile Rh-123 stock solution to an initial row and serial dilutions in the subsequent rows of cells with an automatic dispensing device to obtain the desired range of concentrations for each experiment. Each plate had one row of cells not exposed to Rh-123 that served as control cultures. Cytotoxicity determinations were done daily over a period of 1–8 days of Rh-123 exposure. Two microtiter plates were taken for each time point tested, with one used for immediate viability assessment and the other for testing the ability of Rh-123-treated cells to recover and grow following termination of exposure. For this, medium from wells of the treated plates was completely removed and the wells washed with serum-free medium before incubation with fresh 10% FBS containing DMEM/F12 culture medium for a subsequent 2–5 days before subjecting to cell viability determinations.

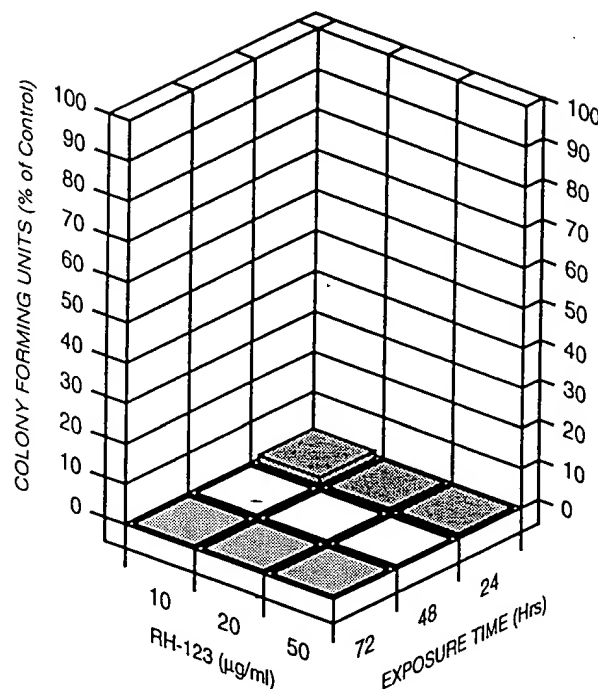
### Determination of Viability of Cells

Determination of viability of cells in the microtiter plates was done by incubation of the cells with 0.4 mg/ml MTT for 4 hours at 37°C, subsequent removal of the medium, and dissolving the cell bound dye in 150 µl DMSO. The plates were next read at  $A_{540}$  nm with an Emax precision microplate reader (Molecular Devices

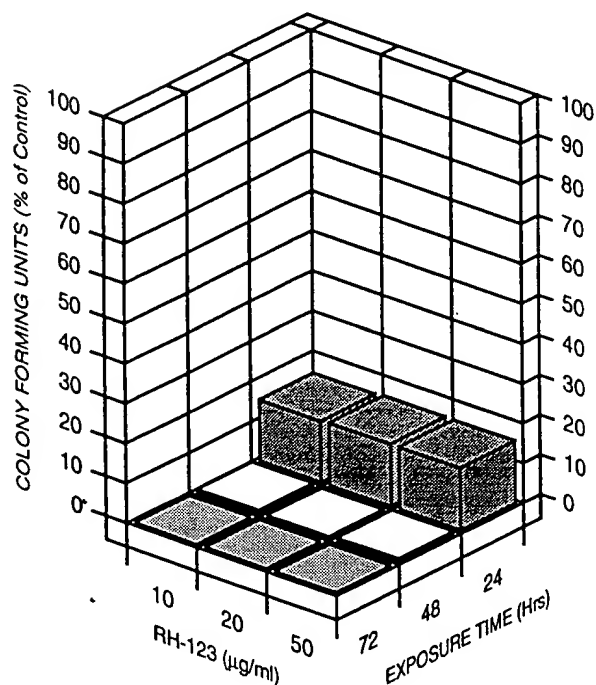
(a) NPF-209



(b) PC-3



(c) DU-145



(d) LNCaP

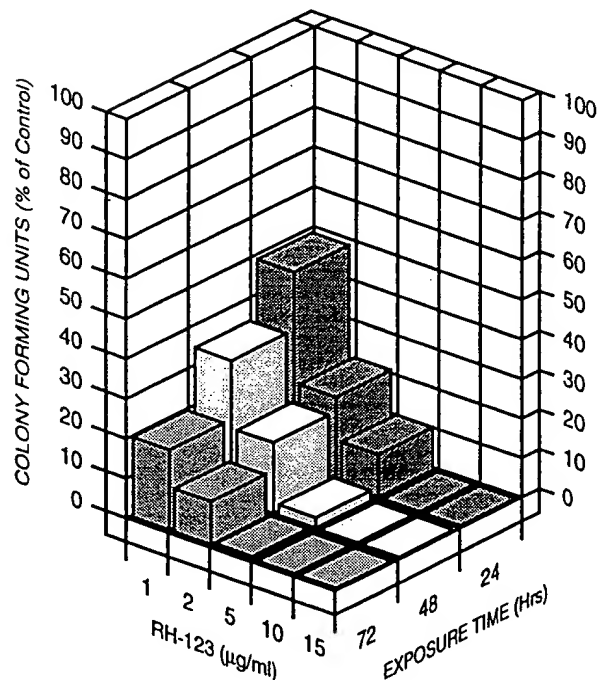


Fig. 4 a: The effect of Rhodamine 123 on colony growth in NPF non-tumorigenic prostate cells. Data shown as relative number of colonies following exposure to 5, 10, and 15  $\mu\text{g/ml}$  Rh-123 for 24, 48, or 72 hrs. Number of colonies in treated dishes were expressed as percent of colonies detected in control, untreated sister cultures. See Materials and Methods for details of procedures used. b: The effect of

Rhodamine 123 on colony growth in PC-3 prostate cancer cells. Other details are as in A. c: The effect of Rhodamine 123 on colony growth in DU145 prostate cancer cells. Other details are as in A. d: The effect of Rhodamine 123 on colony growth in LNCaP prostate cancer cells. Other details are as in a.

Corporation, Menlo Park, CA). MTT is reduced to an insoluble formazan by mitochondria in living cells. The cell bound dye is dissolved with DMSO and spectrophotometrically quantitated by absorbance at 540 nm. The OD<sub>540</sub> nm reading is a measure of the number of viable cells present in the test sample. Growth inhibition due to Rh-123 treatment was determined relative to readings obtained with control (untreated) culture wells on each microtiter plate. Results were confirmed by two to three repeat experiments with each cell line.

#### Determination of Rh-123 Retention by Cells In Vitro

Rh-123 retention by the cells used in this study was determined by flow cytometry utilizing an EPICS Profile II Flow Cytometer (Coulter Corp., Miami, FL). Subconfluent cell cultures were exposed to Rh-123 for 1 hour, washed, and incubated for 24 hours in Rh-123-free culture medium. Rh-123 uptake and retention was determined by comparing fluorescence intensities (at an excitation wavelength of 488 nm) of 10,000 cells collected immediately after 1-hour Rh-123 exposure and 24 hours after termination of Rh-123 exposure.

### RESULTS

#### Rat Prostate Adenocarcinoma

No gross changes were noted in the tumor mass of the prostate complex. Microscopic examination of the treated rats' prostate complexes revealed tumor tissue with marked cellular and acinar destruction, pyknosis, cytoplasmic smearing, and intraepithelial cyst formation (Fig. 1). The tumor mass had not decreased in size presumably because of the accumulated debris of dead and dying cells. Normal surrounding tissue showed no change. Rh-123 was found to be a potent antitumor drug without causing adverse effects on normal tissue.

#### Toxicity

Three of five mice died at a dose of 20 mg/kg Rh-123 in DMSO; one of five mice died at 15 mg/kg Rh-123 in DMSO. Two of five mice died that received only 50% DMSO. If the alcohol-glucose solution was used as a diluent, mice tolerated a dose of 20 mg/kg of Rh-123 given every other day for a period of 2 weeks with no mortality.

#### Rh-123 Cytotoxicity on Cells In Vitro

Figures 2 and 3 are representative of the data obtained in repeat experiments with the different cells studied following exposure to 0–80 µg/ml of Rh-123 for a period of 1–7 days. Figure 2 compares the cytotoxic effects observed with the different cells over a broad (0–80 µg/ml) range of Rh-123 concentrations; Figure 3 shows data from a different experiment covering a narrower range (0–16 µg/ml) of Rh-123 concentrations.

TABLE I. Retention of Rh-123 After 24 Hours in Dye-Free Medium\*

Cell line	% Retention of Rh-123
Human prostatic carcinoma	
DU 145	23
LNCaP	
Population 1	24
Population 2	64
PC-3	40
Normal prostate fibroblast	
NPF-209	9

\*Relative dye retention was estimated from the shift in mean fluorescence after Rh-123-labeled cells were allowed to recover in dye-free medium for 24 hours. Mean fluorescence values were normalized to 100% at 1 hour labeling with 10 µg/ml Rh-123.

The loss of viable cells was marked in all three types of cancer cells (PC-3, LNCaP, and DU 145) exposed for 2 days or longer to Rh-123 at concentrations as low as 1.25–2 µg/ml; in contrast, viability of NPF diploid cells remained relatively high even after prolonged Rh-123 exposures (Figs. 2, 3). Extent of cytotoxic effects was somewhat different in the three prostate cancer cell lines; PC-3 cells appeared to be the most sensitive to Rh-123 exposure; DU 145 cells appeared to be more refractory than either PC-3 or LNCaP (Figs. 2, 3). There were no significant increases in cytotoxic effects on cells at Rh-123 concentrations higher than 10 µg/ml (Fig. 2). Recovery of Rh-123-treated prostate cancer cells incubated in Rh-123-free, normal growth medium was poor; the proportionate loss in viable cells remained the same or became more acute, suggesting continued loss of viable cells or inhibition of growth, despite restoration to normal growth media (Fig. 2).

Clonogenic assays (Fig. 4) showed that colony growth of PC-3 and LNCaP cells was greatly suppressed following exposure to 10 µg/ml of Rh-123. Growth suppression due to Rh-123 was less marked in DU 145 cells. Complete suppression of colony growth in all three cancer cell lines was noted following Rh-123 10 µg/ml exposure for 72 hours. There was less dose-dependent suppression of normal human adult prostate fibroblasts with maximal suppression (28% of control) following a 72-hour exposure. Total suppression was not achieved even after a 72-hour exposure to 50 µg/ml (data not shown).

The above data correlated well with Rh-123 retention obtained by flow cytometry (Table I). Significant amounts of Rh-123 (73–64%) were retained by the three cancer cell lines, 24 hours after withdrawal of Rh-123. In contrast, over 90% of the Rh-123 taken up by NPF cells was lost within 24 hours following removal of the drug. Thus, these data suggest that the increased toxicity of Rh-123-treated prostate cancer cells observed is due to their selective retention of the drug.

## DISCUSSION

Although the destructive effect of Rh-123 on cells in vitro has been reported by several authors [3,12-14] there are few reports of its effect on in vivo solid tumors [15]. Our previously reported studies [4,5], as well as a large series of rats with transplantable tumors treated successfully with Rh-123 (Arcadi, unpublished data), indicate that Rh-123 can destroy transplanted prostate tumors in rats. The studies presented here demonstrated cell destruction by Rh-123 in an autochthonous rat prostate adenocarcinoma with a dose given for a short duration, and also, the preferential sensitivity of three different prostate cancer cell lines.

These studies support the thesis that Rh-123 may be an effective agent for the treatment of metastatic, hormone refractory prostate cancer. Animal studies have demonstrated efficacy and acceptable toxicity.

## ACKNOWLEDGMENTS

The authors wish to thank the Special Rhodamine Group of Pasadena for generous support. We acknowledge, with thanks, the assistance of Dr. Morris Pollard in providing the Lobund-Wistar rats that were used in this experiment.

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## EDITORIAL COMMENTS

The study reported by Arcadi and co-workers is a bit of a "good news, bad news" story. The "good news" is that this study addresses a profoundly deficient area—the development of effective chemotherapies for androgen-insensitive prostatic adenocarcinomas and the means to measure their effectiveness rapidly. There is a clear need for the development of assay systems that can monitor, and predict, in vivo sensitivity of tumor cells to therapeutic intervention. While flow cytometric measurements have been proposed for some time [1,2] only recently has the realization that tissue culture systems provide an inadequate model for the study of drug retention and tumor cell killing in vivo [3] begun to have an impact on our thinking. While the use of flow cytometric (or other assays) that can potentially measure tumor cell drug sensitivity looks promising, this area clearly needs well-defined standards and standardized methodologies before it can become clinically useful. The measurement of a certain percentage reduction in the retention of Rh-123 as measured here or any potentially chemotoxic agent is meaningless by itself. These values must be reported in molecules of drug retained per cell (with some understanding of the distribution variation within the population; i.e., is it a 2-fold or 200-fold difference within the tumor?), using standardized methodologies proved useful to predict log kill of tumor cells in vivo. When (and if) we arrive at that point, measurements of drug uptake and retention have a realistic potential to provide clinically useful information on individual cancer patients' tumors.

The bad news is that it involves a comparison of apples and oranges. Here, the connection between rat prostatic adenocarcinoma growing (or not growing) in vivo following Rh-123 (or any drug) treatment, and the toxicity of the same agent on human prostate cancer cell lines growing in vitro is tenuous at best. To demonstrate that Rh-123 suppresses cell growth in tissue culture and to suggest that this supports "the thesis that Rh-123 may be an effective agent for the treatment of metastatic hormone-refractory prostate cancer" (see Discussion) is a connection that has too often failed in the past. As pointed out by Tannock [4] and many others, the local environment of solid malignancies in situ has a profound impact on the responsiveness or nonresponsiveness of cancers where they really count to a patient in his or her body.

T. Vincent Shankey, PhD  
Departments of Urology and Pathology  
Loyola University Medical Center  
Maywood, IL 60153

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#### REPLY TO EDITORIAL COMMENTS

We completely agree with the comments. However, we must all remind ourselves that a project must start at

some point—our in vitro studies are at a starting, but we believe a significant, phase. We feel our in vivo studies of an autochthonous rat prostate complex adenocarcinoma which show significant cell destruction are unsailable. The commentator, unfortunately, does not comment on the most important feature of Rh-123. It acts by preferentially destroying tumor cell mitochondria with minimal toxicity to normal cells.

—John A. Arcadi, MD

## **EXHIBIT B**

# Synthesis and Evaluation of Novel Rhodacyanine Dyes That Exhibit Antitumor Activity

Masayuki Kawakami,\*<sup>†</sup> Keizo Koya,<sup>†</sup> Toshinao Ukai,<sup>†</sup> Noriaki Tatsuta,<sup>†</sup> Akihiko Ikegawa,<sup>†</sup> Keizo Ogawa,<sup>†</sup> Tadao Shishido,<sup>†</sup> and Lan Bo Chen<sup>‡</sup>

Ashigara Research Laboratories, Fuji Photo Film Company, Ltd., 210 Nakanuma, Minamishigara, Kanagawa 250-01, Japan, and Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115

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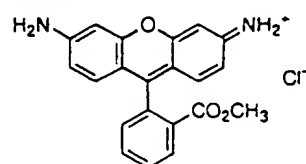
Rhodacyanine dyes and several analogous delocalized lipophilic cations (DLCs) were synthesized and evaluated as novel antitumor agents. Rhodacyanine dye consists of two heteroaromatic rings such as thiazoles at both termini of the conjugate systems and 4-oxothiazolidine (rhodanine) in the middle of it. Compounds with such a unique double-conjugate structure were found to inhibit the growth of several tumor cell lines, such as colon carcinoma CX-1, and to exhibit relatively low toxicity against normal kidney cell line CV-1 (e.g.,  $IC_{50}(CX-1) = 50$  nM,  $IC_{50}(CV-1) = 17.3$   $\mu$ M; selectivity index = 346 for compound 5). These compounds were also found to be efficacious in the tumor-bearing nude mice model (e.g., against human melanoma LOX; T/C (%) = 168 for compound 5). Structural modifications on rhodacyanine, including deletion of a heteroaromatic ring involved in the merocyanine conjugate system and replacement of rhodanine with a structurally related moiety such as 4-oxoimidazolidine or 4-oxo-1,3-dithiolane, resulted in a loss of the selectivity and/or the activity. Our current structure-activity studies imply that the double-conjugate system with a rhodanine moiety is essential for the selective activity of rhodacyanine dyes, and we find this class of compounds as unique antitumor agent candidates.

## Introduction

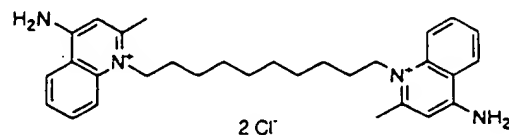
Mitochondria carry out most cellular oxidations and produce the animal cell's ATP. In mitochondria the energy available from combining oxygen with reactive electrons carried by NADH pumps out protons from the inner membrane, and the energy is thus stored in the electrochemical proton gradient which consists of a membrane potential and a pH gradient. The resultant transmembrane gradient is in turn used to synthesize ATP. The membrane potential has been monitored by organic compounds known as membrane potential sensitive probes.<sup>1-3</sup> These probes, most of which are the positively charged organic compounds, are taken into cells in response to the high negative charge in the mitochondria, where they can be toxic preferentially. Since the membrane potential of the mitochondria in tumor cells is higher than that of normal cells, these compounds are accumulated in the tumor mitochondria.<sup>4-6</sup> Therefore, cytotoxic  $\pi$  electron-delocalized lipophilic cations (DLCs) are proposed to kill tumor cells selectively, and DLCs including rhodamine 123,<sup>4-7</sup> dequalinium,<sup>8</sup> thiacyanines,<sup>9,10</sup> and thiopyrylium AA-1 (Chart 1)<sup>11</sup> have been explored as potential antitumor drugs. In spite of high potential as antitumor agents, none of them have met the criteria for clinical development, such as water solubility, stability, toxicity, and pharmacokinetics.

In an effort to find novel DLCs, we have screened a wide variety of DLCs from the compound library at Fuji

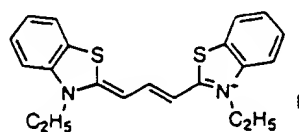
Chart 1



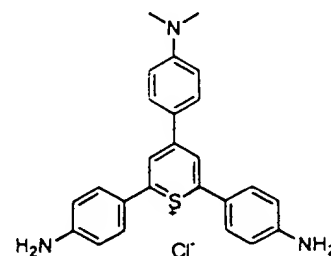
Rhodamine 123



Dequalinium



Thiacyanine (S23)



Thiopyrylium (AA-1)

Photo Film Co., Ltd. which was developed for photographic systems. Rhodacyanine dyes that were originally studied as silver halide sensitizers showed high inhibitory effect *in vitro* on the growth of tumor cells. In this paper we focus on synthesis and evaluation of

\* Fuji Photo Film Co., Ltd.

<sup>†</sup> Harvard Medical School.

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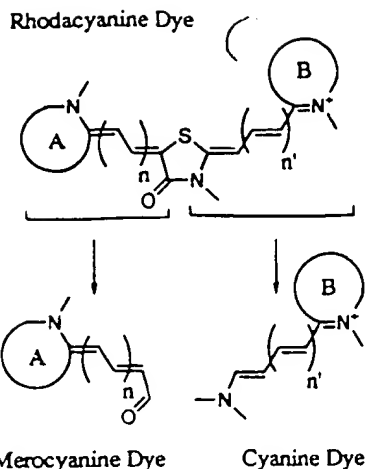
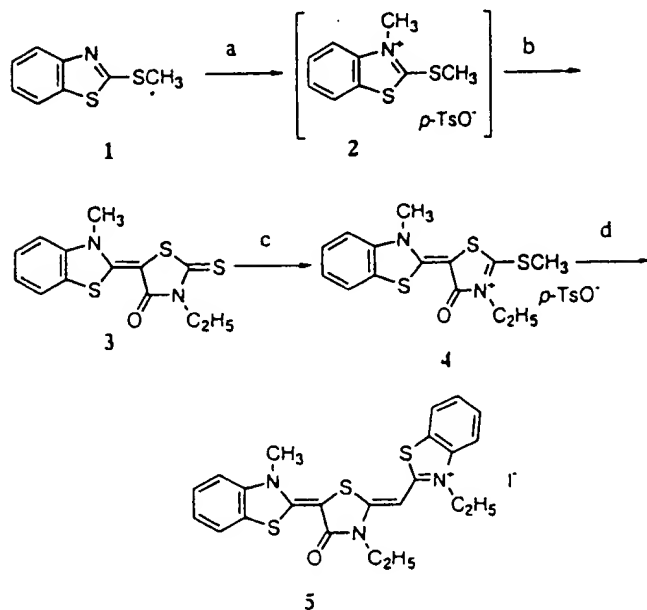


Figure 1. General formula of rhodacyanine dye and its component parts: merocyanine dye and cyanine dye.

#### Scheme 1<sup>a</sup>



<sup>a</sup>  $p\text{-TsO}^- = p\text{-CH}_3\text{-C}_6\text{H}_4\text{-SO}_3^-$

<sup>a</sup> Reagents: (a) methyl *p*-toluenesulfonate/anisole; (b) 3-ethyl-4-oxothiazolidine-2-thione/ $\text{NEt}_3/\text{MeCN}$ ; (c) methyl *p*-toluenesulfonate/DMF; (d) 3-ethyl-2-methylbenzothiazolium iodide/ $\text{NEt}_3/\text{MeCN}$ .

novel rhodacyanine dyes and their analogs to clarify structure-activity relationships.

#### Chemistry

Rhodacyanine dye consists of three rings, two heteroaromatic rings and a central 4-oxothiazolidine (rhodanine), as represented in Figure 1. At the rhodanine moiety, two dye conjugate systems, neutral merocyanine with heteroaromatic ring A and cationic cyanine with heteroaromatic ring B, are integrated. In the present study, we synthesized two types of rhodacyanine dyes, ( $n = 0, n' = 0$ ) and ( $n = 1, n' = 0$ ) in Figure 1, according to the reported procedures<sup>12</sup> with some modifications. Typical synthesis examples are illustrated in Schemes 1 and 2, respectively. In both cases, merocyanine dyes (3 and 8) were synthesized first followed by the conjugation with cyanine units.

*N*-Methylation of 2-(methylthio)benzothiazole (1) using methyl *p*-toluenesulfonate gave 2, which was con-

densed with 3-ethyl-4-oxothiazolidine-2-thione to give merocyanine dye 3. *S*-Methylation of 3 was followed by the reaction of 3-ethyl-2-methylbenzothiazolium iodide in the presence of triethylamine to give a rhodacyanine dye (5). Other rhodacyanine dyes ( $n = 0, n' = 0$ ) were also synthesized in an analogous manner, and the results are summarized in Table 1.

3-Ethyl-4-oxothiazolidine-2-thione (6) was reacted with 1,3-diaza-1,3-diphenylpropene and then with acetic anhydride to give compound 7, which was condensed with 3-ethyl-2-methylnaphtho[1,2-*d*]thiazolinium iodide to give merocyanine dye 8. A rhodacyanine dye (10) was synthesized from merocyanine intermediate 8 under similar conditions as those for the synthesis of 5 from 3. Other rhodacyanine ( $n = 1, n' = 0$ ) dyes were also synthesized in an analogous manner, and the results are summarized in Table 1.

Because the rhodanine moiety is conjugated with two double bonds, there are several possible geometrical isomers around this moiety. However, our NMR studies suggested that these rhodacyanine dyes exist as a single isomer. As illustrated in Figures 2 and 3, X-ray crystallographic studies for compounds 32 and 33 revealed that in the merocyanine unit *N*-substituents were situated on the opposite side of methine carbons, while in the cyanine unit *N*-substituents were sitting on the same side for both cases. As for the geometrical isomerism of the merocyanine moiety of 33, it was determined to be the *s*-trans configuration (Figure 3).

For structure-activity studies, several DLC compounds analogous to rhodacyanines, including analogs whose heteroaromatic ring A was replaced with other moieties (13a,b, 14, 15, 23) and those whose rhodanine moiety was substituted with other structurally related moieties (20, 26, 28, 30), were synthesized. The results are summarized in Tables 2 and 3.

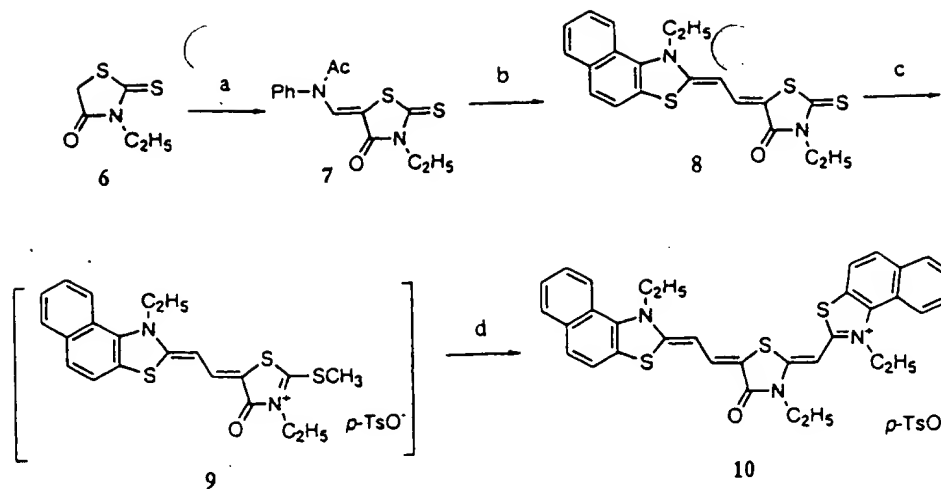
Synthesis of cyanine dye 13b was accomplished by the reaction of 3-ethyl-2-methylbenzothiazolium iodide (11) with ethyl thiocyanate followed by the reaction of bromoacetic acid as illustrated in Scheme 3.<sup>13</sup> Treatment of 11 with phenyl thiocyanate gave *N*-phenyl derivative 13a, which was converted to 14 and 15 by the reaction of 1,3-diaza-1,3-diphenylpropene and 1,5-diaza-1,5-diphenyl-1,3-pentadiene, respectively.

A rhodanine analog (20) was synthesized according to the reported procedures as illustrated in Scheme 4.<sup>14,15</sup> Carbodithioate 16<sup>14</sup> was treated with triethyl orthoformate and then 3-ethyl-2-methylbenzoxazolium iodide to give merocyanine dye 18, which was further converted to the target compound 20 under similar conditions as those for the synthesis of rhodacyanine dye 10.

Other analogs of the rhodanine moiety (26 and 28) containing 4-oxoimidazolidine were synthesized by reported procedures similar to those for the synthesis of the corresponding rhodacyanine dye.<sup>16</sup> The other rhodanine analog (30) containing 4-oxo-1,3-dithiolane<sup>16</sup> and cyanine dyes (21 and 22)<sup>10</sup> were also synthesized by reported procedures, respectively.

#### Biological Results and Discussion

**In Vitro Clonogenic Assay.** The compounds synthesized here were evaluated for their inhibitory effects on the growth of human colon carcinoma cell (CX-1) and toxicity against normal epithelial cell (CV-1). In each



<sup>a</sup> Reagents: (a) (i) 1,3-dithiane-1,3-diphenylpropene/ligroin, (ii)  $\text{Ac}_2\text{O}/\text{NEt}_3$ ; (b) 3-ethyl-2-methylnaphthol[1,2-d]thiazolium *p*-toluenesulfonate/ $\text{Ac}_2\text{O}/\text{NEt}_3/\text{MeCN}$ ; (c) methyl *p*-toluenesulfonate/ $\text{DMF}/\text{toluene}$ ; (d) 3-ethyl-2-methylnaphthol[1,2-d]thiazolium *p*-toluenesulfonate/ $\text{NEt}_3/\text{MeCN}$ .

Table 1. Structure of Rhodacyanine Dyes

Compd	$a_1, a_2$	$a_3$	$R_1$	$\alpha$	$R_2$	$b_1$	$b_2, b_3$	$X^-$
5	H, H	S	$\text{CH}_3$	0	$\text{C}_2\text{H}_5$	S		$\Gamma$ $\text{AcO}^-$
24	H, H	S	$\text{CH}_3$	0	Ph	S		<i>p</i> - $\text{TsO}^-$
32	H, H	S	$\text{CH}_3$	0		O		$\text{Cl}^-$
10		S	$\text{C}_2\text{H}_5$	1	$\text{C}_2\text{H}_5$	S		<i>p</i> - $\text{TsO}^-$
25	H, H	S	$\text{C}_2\text{H}_5$	1	Ph	S		$\Gamma$
27		S	$\text{C}_2\text{H}_5$	1	$\text{C}_2\text{H}_5$	S		<i>p</i> - $\text{TsO}^-$
29	H, H	O	$\text{C}_2\text{H}_5$	1	$\text{CH}_3$	S		$\Gamma$
33	$\text{CF}_3, \text{H}$	S	$\text{C}_2\text{H}_5$	1	$\text{C}_2\text{H}_5$	S	H, $\text{CH}_3$	$\Gamma$

case, the cells were incubated with a compound for 24 h and then in compound-free medium for 2 weeks. The number of colonies was measured by a crystal violet-staining method, and the results are expressed as the  $\text{IC}_{50}$  value.

Cyanine dyes such as thiacyanine (S23)<sup>9</sup> and other DLCs<sup>4-8,11</sup> were reported to exhibit selective activity against carcinoma cells. In this study two cyanine dyes with different methine lengths, 21 (S23) and 22, showed high inhibitory effect on CX-1 with  $\text{IC}_{50}$  values of 60 nM (Table 4). As a part of structure modifications of cyanine dyes in an attempt to find a novel class of DLCs with higher activity and selectivity, a methine unit of the cyanine dyes was replaced with a

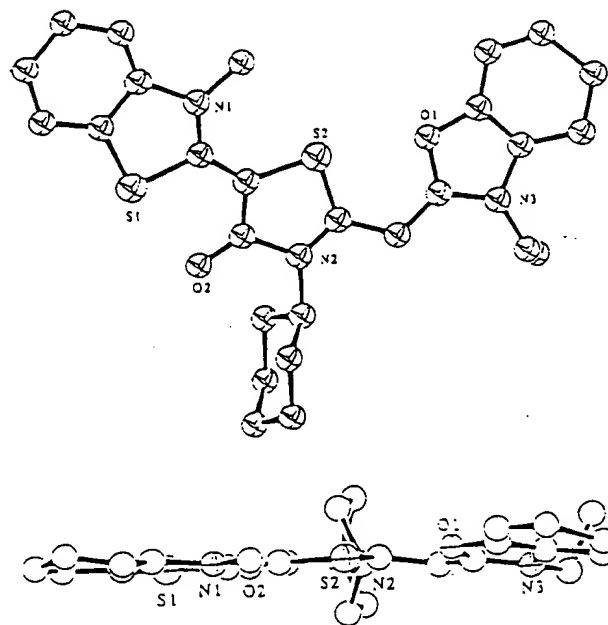


Figure 2. X-ray-determined structure of rhodacyanine dye 32.

rhodanine moiety. The rhodacyanine dyes thus synthesized were found to be equipotent (5) or 1.6 times more potent (10) to their cyanine counterparts as depicted in Table 4. Another interesting finding about rhodacyanines in Table 4 is their low toxicity against CV-1 and the resulting high selectivity defined by  $\text{IC}_{50}(\text{CV-1})/\text{IC}_{50}(\text{CX-1})$ : they were more selective than the corresponding cyanines by 17-fold (5 vs 21) and 109-fold (10 vs 22).

To understand why rhodacyanine dyes have such a high activity specifically against tumor cells, our first structure-activity study focused on deletion or replacement of the heteroaromatic ring of the merocyanine conjugate system (ring A in Figure 1). Simple deletion of the *N*-ethylbenzothiazolidene group of rhodacyanine 5 led to a decrease of the activity by 26-fold (5 vs 13b in Table 5). Replacement with a cyclohexylidene group (23) resulted in further loss of activity. While compounds 13b and 23 still retain some moderate activities, it may be due to the delocalized cationic cyanine

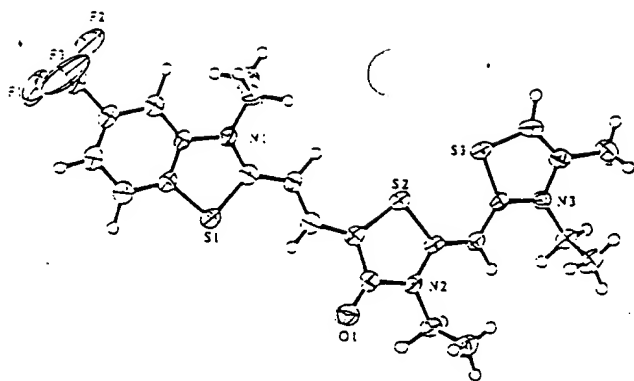


Figure 3. X-ray-determined structure of rhodacyanine dye 33.

Table 2. Structure of Rhodacyanine Dye Analogs

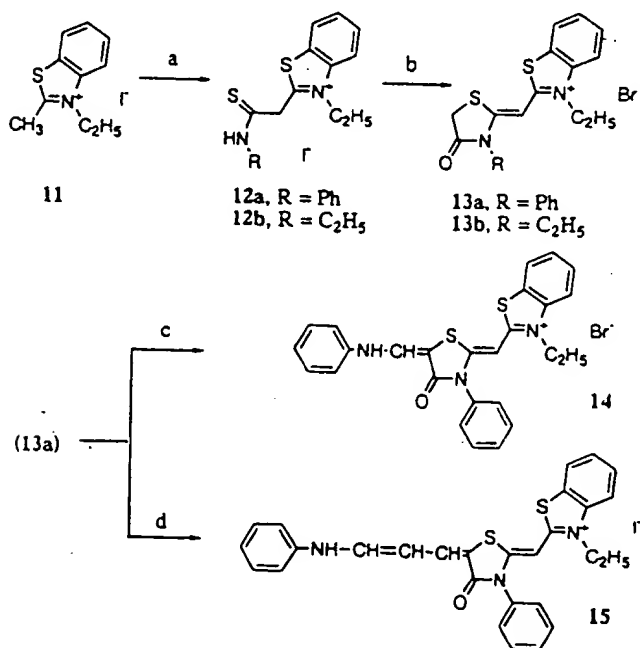
Compd	R <sub>1</sub> , R <sub>2</sub>	R <sub>3</sub>	X <sup>-</sup>
13a	H, H	Ph	Br <sup>-</sup>
13b	H, H	C <sub>2</sub> H <sub>5</sub>	Br <sup>-</sup>
14		Ph	Br <sup>-</sup>
15		Ph	I <sup>-</sup>
23		C <sub>2</sub> H <sub>5</sub>	<i>p</i> -TsO <sup>-</sup>

conjugate system (between rhodanine and ring B) in these compounds. The fact that relatively low selectivity of these compounds (30 for 13b, 9 for 23) compared to a rhodacyanine (346 for 5) are within the same range for cyanine dyes 21 and 22 in Table 4 supports this hypothesis. The results of *N*-phenyl derivatives were quite similar to those of *N*-ethyl derivatives (Table 5). Deletion (13a) or replacement (14, 15) of the benzothiazolidene moiety of rhodacyanine 24 made the activity and selectivity as low as those for 13b and 23. In contrast, elongation of the methine unit between the A ring and rhodanine moiety in rhodacyanine dye (from *n* = 0 to *n* = 1 in Figure 1) affected the quite selective activity of 24 only in a moderate way, and the resultant

Table 3. Structure of Rhodacyanine Dye Analogs

Compd	a <sub>1</sub> , a <sub>2</sub>	a <sub>3</sub>	R <sub>1</sub>	n	C	b <sub>1</sub> , b <sub>2</sub>	X <sup>-</sup>
20	H, H	O	C <sub>2</sub> H <sub>5</sub>	1		H, H	I <sup>-</sup>
26	H, H	S	CH <sub>3</sub>	0		H, H	I <sup>-</sup>
28		S	C <sub>2</sub> H <sub>5</sub>	1			I <sup>-</sup>
30	H, H	O	C <sub>2</sub> H <sub>5</sub>	1		H, H	I <sup>-</sup>

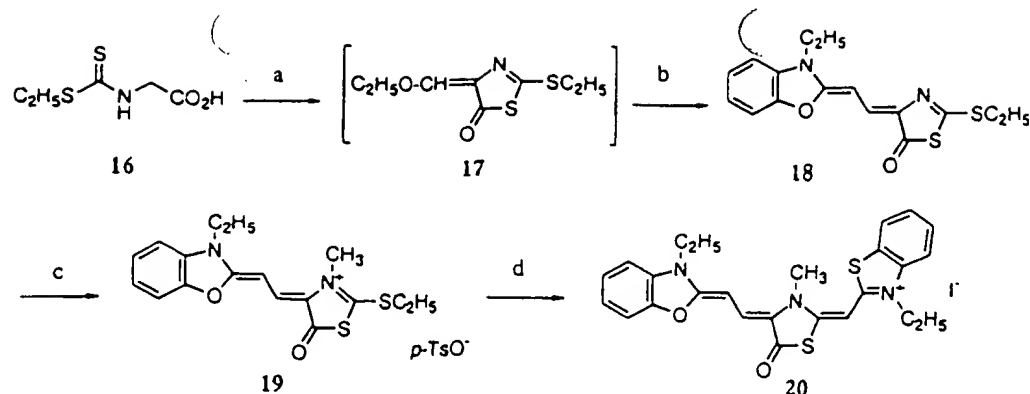
Scheme 3<sup>a</sup>



<sup>a</sup> Reagents: (a) 12a—phenyl thiocyanate/NaH/THF, 12b—ethyl thiocyanate/pyridine/NEt<sub>3</sub>; (b) 13a—bromoacetic acid/1-BuOH, 13b—bromoacetic acid/acetic acid; (c) 1,3-diphenylpropene/ethylene glycol; (d) 1,5-diphenyl-1,3-pentadiene-HCl salt/NEt<sub>3</sub>/NaI/MeOH.

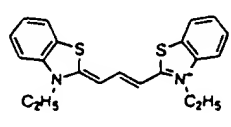
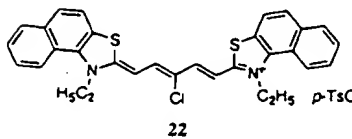
compound 25 showed high activity and selectivity (IC<sub>50</sub> against CX-1 = 60 nM, selectivity = 50).

An attempt to measure the IC<sub>50</sub> values for merocyanine dyes such as 3 failed due to their poor solubility. In our preliminary experiments, however, they did not exhibit any appreciable activity at 3 μM. A comparison of 3 (merocyanine), 5 (rhodacyanine), and 13b (cyanine), together with other results in Table 5, led us to the hypothesis that the merocyanine conjugate system in rhodacyanine dyes enhanced moderate activity which



<sup>a</sup> Reagents: (a)  $\text{CH}(\text{OEt})_2/\text{Ac}_2\text{O}$ ; (b) 3-ethyl-2-methylbenzoxazolium iodide/ $\text{NEt}_3/\text{EtOH}$ ; (c) methyl *p*-toluenesulfonate; (d) 3-ethyl-2-methylbenzothiazolium iodide·HCl salt/ $\text{NEt}_3/\text{EtOH}$ .

**Table 4.** Clonogenic Assay of Rhodacyanine Dyes and Cyanine Dyes on Human Colon Carcinoma (CX-1) and Monkey Normal Kidney Epithelial Cells (CV-1)

Compd	IC <sub>50</sub> (μM)		Ratio
	CX-1	CV-1	
	0.06	1.2	20
	0.06	0.1	2
5	0.05	17.3	346
10	0.03	6.5	217

**Table 5.** Clonogenic Assay of Rhodacyanine Dyes and Their Cyanine Dye Analogs

compd	IC <sub>50</sub> (μM)		ratio CV-1/CX-1
	CX-1	CV-1	
5	0.05	17.3	346
24	0.01	2.2	220
25	0.06	3.0	50
13a	2.3	46.1	20
13b	1.3	38.9	30
14	1.5	1.5	1
15	0.8	9.8	12
23	1.8	16.2	9

arised from the cationic cyanine conjugate system in a selective manner.

To obtain further insights into the role of the merocyanine conjugate system, we modified the structure of the rhodanine moiety. Replacement with 4-oxoimidazolidine resulted in a decrease of the activity against CX-1 cells (5 vs 26, 27 vs 28 in Table 6). Compound 28 was more toxic against CV-1 than the corresponding rhodacyanine 27 by 33-fold, resulting in the marked decrease of the selectivity (66-fold). Analysis of Table 6 reveals that an analog with the 4-oxo-1,3-dithiolan moiety (30) exhibited further decreased activity (>50 times), while substitution with 5-oxothiazoline (20) made the activity only one-half that of the parent compound (29). Thus, our structure-activity studies so far concluded that the rhodanine moiety is indispensable for the quite high and selective activity for tumor cells.

**Table 6.** Clonogenic Assay of Rhodacyanine Dyes and Their Analogs of the 4-Oxothiazolidine (Rhodanine) Moiety

compd	IC <sub>50</sub> (μM)		ratio CV-1/CX-1
	CX-1	CV-1	
5	0.05	17.3	346
27	0.05	26.2	524
29	0.03	1.7	57
20	0.07	3.4	49
26	1.3	25.4	20
28	0.1	0.8	8
30	>1.7	3.4	<2

**Table 7.** Clonogenic Assay on Four Human Cancer Cell Lines<sup>a</sup>

compd	anion	IC <sub>50</sub> (μM)			
		EJ	LOX	MFC-7	CRL1420
5	I <sup>-</sup>	0.07	0.02	0.09	0.05
31	AcO <sup>-</sup>	0.06	0.01	0.4	0.06
24	<i>p</i> -TsO <sup>-</sup>	0.04	0.04	0.06	0.04
25	I <sup>-</sup>	0.4	0.04	0.04	0.07

<sup>a</sup> EJ, bladder carcinoma; LOX, melanoma; MCF-7, breast carcinoma; CRL1420, pancreatic carcinoma.

Our physical chemistry studies including X-ray crystallography, absorption and NMR spectra, and molecular orbital calculations revealed that all three rings (A, B, rhodanine) were almost coplanar and that  $\pi$  electrons delocalized throughout the molecule. This electron delocalization across the two dye conjugate systems was partly indicated from a marked bathochromic shift of rhodacyanine 5 ( $\lambda_{\text{max}}$ : 500.0 nm in methanol) compared to the partial structured merocyanine 3 ( $\lambda_{\text{max}}$ : 428.0 nm in methanol) and cyanine 13b ( $\lambda_{\text{max}}$ : 390.4 nm in methanol). A molecular orbital calculation suggested that the p orbital of rhodanine's sulfur atom was involved in such an electron delocalization, which was thought to be a key factor for the selective activity of rhodacyanine dyes against tumor cells. Further studies concerning the relationship between physical property and activity are in progress.

Further *in vitro* studies of rhodacyanines 5, 31, 24, and 25 were conducted using four human tumor cell lines: EJ (human bladder carcinoma), LOX (human melanoma), MCF-7 (human breast carcinoma), and CRL-1420 (human pancreatic carcinoma). As summarized in Table 7, all the compounds showed high inhibitory activities against the cell lines with IC<sub>50</sub> values of 10–90 nM, suggesting that rhodacyanine dyes are potential medications for a wide spectrum of tumors. The highly water-soluble compound 31 (solubility > 10

compd	treatment	in vivo activity			
		T/C <sup>a</sup> (%)		TI <sup>b</sup> (%) CX-1	P <sup>c</sup>
		LOX	OVCARIII		
5	at 5 mg/kg ip on days 1, 5, 8, 12, and 15	168			<0.05
31	at 5 mg/kg ip on days 1, 3, 7, 10, and 16		180		<0.01
	at 2 mg/kg ip on days 1, 2, 4, 5, 7, 8, and 10	156			<0.05
	at 4 mg/kg ip on days 1, 2, 4, 5, 7, 8, and 10	163			<0.01
24	at 5 mg/kg ip on days 1, 5, 8, and 12	183			<0.01
	at 5 mg/kg ip on days 1, 3, 7, 10, and 16		>300		<0.05
	at 5 mg/kg ip on days 1, 5, and 7			42	
25	at 1.5 mg/kg ip on days 1, 3, 4, 7, and 9	256			<0.05

<sup>a</sup> T/C represents the ratio of the median survival time of drug-treated to control, untreated tumor-bearing mice, expressed as a percentage. Each group consisted of five nude mice. <sup>b</sup> TI represents tumor inhibition ratio, which was evaluated at day 11 and calculated as follows: inhibition ratio (%) =  $(A - B)/A \times 100$ , where A is the average tumor weight in the control group and B is that in the treated group. Each group consisted of five nude mice. <sup>c</sup> The statistical significance of difference between the control and the drug-treated group was determined by applying the Bartlett's, Dunnett's, and Scheffé's test.

mg/mL), which has an acetate anion and was prepared from the corresponding less soluble iodide 5 (solubility < 0.1 mg/mL) using an anion-exchange method, has similar IC<sub>50</sub> values to those for 5, which implies that the cation dye is the active component while the counteranion has little influence upon activities.

**In Vivo Antitumor Activities.** Rhodacyanine dyes 5, 31, 24, and 25 were evaluated for their inhibitory activities against the growth of human cell lines in the nude mice model. After intraperitoneal (ip) implantation of  $2 \times 10^6$  human melanoma LOX cells (to which the compounds exhibited the highest activity in Table 7), male Swiss nu/nu mice had a median survival time of 24 days. In contrast, mice that received ip administration of compound 5 at 5 mg/kg on days 1, 5, 8, 12, and 15 showed a median survival time of 40 days with tumor/control (T/C) = 168% (Table 8). Administration of other compounds with appropriately determined doses and schedules made the survival period similar or even longer: T/C = 156% (2 mg/kg) or 163% (4 mg/kg) for 31, 183% for 24, 256% for 25. Compounds 5 and 24 were also tested against human ovarian carcinoma OVCARIII, and both were found to be quite efficacious (T/C = 180% for 5, >300% for 24), and compound 24 in the human colon carcinoma CX-1 nude mice model displayed marked inhibition of tumor growth (tumor inhibition ratio (TI) = 42%). Thus simple anion exchange had little influence upon *in vivo* antitumor efficacy and was a useful method to increase the water solubility which was an important criterion for clinical use. Any significant body weight loss was not observed in these nude mice models, and also the acute toxicity (LD<sub>50</sub>, ip administration into ICR mice) of compound 31 observed was over 30 mg/kg.

## Conclusion

The results described in this study demonstrated that rhodacyanine dyes exhibited selective inhibitory activities *in vitro* against the growth of several human cell lines over CV-1, an indicator cell for normal epithelial cells. They also displayed high-level efficacy against LOX and OVCARIII *in vivo*, with low toxicity. Structure-activity studies indicated that rhodanine was an indispensable moiety for the antitumor activity of rhodacyanines, which was markedly improved from those of cyanines or other DLCs. This class of compounds is expected to be a novel antitumor agent and is being

subjected to further extensive structure-activity studies, results of which will be presented elsewhere.

## Experimental Section

The <sup>1</sup>H-NMR spectra were recorded on a Bruker AMX-600, ARX-300, or AC-200 spectrometer with tetramethylsilane as internal standard. Chemical shifts are given in ppm, coupling constants are in hertz, and splitting patterns are designated as follows: s, singlet; brs, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. Ultraviolet-visible (UV-vis) absorption spectra were recorded on a Shimadzu UV-260 spectrophotometer. Fast atom bombardment (FAB) mass spectra were determined with a JEOL DX303 mass spectrometer, and high-resolution mass spectrometry (HRMS) was recorded on a JEOL SX-102A mass spectrometer; electron ionization (EI) mass spectra were determined with a JEOL JMS-D300 instrument. Elemental analyses were performed on Yanagimoto MT-3 and Dionex 2000i/SP instruments, and the results (C, H, N) were within ±0.4% of theoretical values unless indicated otherwise. Correct elemental analyses for most of the compounds could only be obtained by factoring in partial hydration of these organic salts.

**3-Ethyl-5-(3-methylbenzothiazolin-2-ylidene)-4-oxothiazolidine-2-thione (3).** A mixture of 2-(methylthio)benzothiazole (1) (40.0 g, 220 mmol), methyl *p*-toluenesulfonate (61.5 g, 330 mmol), and anisole (56 mL) was stirred at 120–136 °C for 4 h. After the mixture was cooled to room temperature, 3-ethyl-4-oxothiazolidine-2-thione (6) (35.0 g, 220 mmol) and acetonitrile (800 mL) were added. To this mixture was added triethylamine (36.4 g, 360 mmol) dropwise under 15 °C with constant stirring and cooling, and the resulting mixture was stirred at 10 °C for 4 h. The yellow precipitate was collected and washed with acetonitrile (40 mL) and then with methanol (140 mL). The crude product thus obtained was suspended in acetone (210 mL) and methanol (420 mL), and the mixture was stirred under reflux for 15 min. After cooling to 25 °C, the precipitate was collected and washed with methanol (140 mL) to give 3 (59.0 g, 87.0%) as yellow crystals: UV-vis (MeOH)  $\lambda_{\max}$  428.0 nm ( $\epsilon$  6.62  $\times 10^4$ ); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.20 (t, *J* = 7.2 Hz, 3H), 3.98 (s, 3H), 4.10 (q, *J* = 7.2 Hz, 2H), 7.35 (t, *J* = 8.0 Hz, 1H), 7.52 (t, *J* = 8.0 Hz, 1H), 7.78 (d, *J* = 8.0 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 1H); MS (EI) *m/z* 308. Anal. (C<sub>13</sub>H<sub>12</sub>N<sub>2</sub>OS<sub>2</sub>) C, H, N, S.

**3-Ethyl-5-(3-methylbenzothiazolin-2-ylidene)-2-(methylthio)-4-oxothiazolinium *p*-Toluenesulfonate (4).** A mixture of 3 (58.0 g, 188 mmol), methyl *p*-toluenesulfonate (105.0 g, 564 mmol), and *N,N*-dimethylformamide (58 mL) was stirred at 130–145 °C for 2.5 h. After the mixture cooled to 95 °C, acetone (500 mL) was added. The mixture was further cooled to 25 °C with constant stirring, and the precipitate formed was collected and washed with acetone (150 mL). The crude product thus obtained was suspended in acetone (400 mL), and the mixture was stirred under reflux for 15 min. After cooling to 25 °C, the precipitate was collected and washed with acetone



(150 mL) to give 4 (86.5 g, 93.0%) as orange crystals: UV-vis (MeOH)  $\lambda_{\text{max}}$  420.7 nm ( $\epsilon$  3.68  $\times 10^4$ );  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  1.33 (t,  $J$  = 7.2 Hz, 3H), 2.27 (s, 3H), 3.05 (s, 3H), 4.17 (q,  $J$  = 7.2 Hz, 2H), 4.24 (s, 3H), 7.10 (d,  $J$  = 8.9 Hz, 2H), 7.46 (d,  $J$  = 8.9 Hz, 2H), 7.52 (dd,  $J$  = 8.0, 8.0 Hz, 1H), 7.70 (dd,  $J$  = 8.0, 8.0 Hz, 1H), 8.00 (d,  $J$  = 8.0 Hz, 1H), 8.18 (d,  $J$  = 8.0 Hz, 1H); MS (FAB $^+$ , glycerine) for  $\text{C}_{11}\text{H}_{15}\text{N}_2\text{O}_3\text{S}$   $m/z$  323, (FAB $^-$ , triethanolamine) for  $\text{C}_{11}\text{H}_{15}\text{N}_2\text{O}_3\text{S}$   $m/z$  171. Anal. ( $\text{C}_{21}\text{H}_{22}\text{N}_2\text{O}_4\text{S}_4 \cdot 0.3\text{H}_2\text{O}$ ) C, H, N, S.

**3-Ethyl-2-[[3-ethyl-5-(3-methylbenzothiazolin-2-ylidene)-4-oxothiazolidin-2-ylidene]methyl]benzothiazolium Iodide (5).** To a mixture of 4 (24.7 g, 50 mmol) and 3-ethyl-2-methylbenzothiazolium iodide (15.3 g, 50 mmol) in acetonitrile (250 mL) was added triethylamine (15.2 g, 150 mmol) dropwise at 70  $^\circ\text{C}$ , and the mixture was stirred for 1.5 h at the same temperature. To the reaction mixture was added ethyl acetate (250 mL), and the mixture was cooled to 30  $^\circ\text{C}$  with constant stirring. The orange precipitate was collected and washed with ethyl acetate (125 mL). The crude product thus obtained was dissolved in methanol (80 mL), and then to this solution was added ethyl acetate (250 mL) at 50  $^\circ\text{C}$  with constant stirring. The precipitate was collected and washed with ethyl acetate (110 mL) to give 5 (14.5 g, 50.0%) as orange crystals: UV-vis (MeOH)  $\lambda_{\text{max}}$  500.0 nm ( $\epsilon$  7.49  $\times 10^4$ );  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  1.28 (t,  $J$  = 7.1 Hz, 3H), 1.38 (t,  $J$  = 7.1 Hz, 3H), 4.22 (s, 3H), 4.30 (q,  $J$  = 7.2 Hz, 2H), 4.71 (q,  $J$  = 7.2 Hz, 2H), 6.69 (s, 1H), 7.36 (t,  $J$  = 7.8 Hz, 1H), 7.52–7.58 (m, 2H), 7.70–7.79 (m, 2H), 7.94–7.98 (m, 2H), 8.26 (d,  $J$  = 7.2 Hz, 1H); MS (FAB $^+$ , nitrobenzyl alcohol) for  $\text{C}_{23}\text{H}_{22}\text{N}_3\text{O}_3\text{S}$   $m/z$  452, (FAB $^-$ , glycerine) for  $m/z$  127. Anal. ( $\text{C}_{23}\text{H}_{22}\text{N}_3\text{O}_3\text{S}_3 \cdot 1.5\text{H}_2\text{O}$ ) C, H, N, S.

**5-[(N-Acetyl-N-phenylamino)methylidene]-3-ethyl-4-oxothiazolidine-2-thione (7).** To a solution of 3-ethyl-4-oxothiazolidine-2-thione (6) (25.0 g, 155 mmol) in ligroin (145 mL) was added 1,3-diaza-1,3-diphenylpropene (32.5 g, 166 mmol), and the mixture was stirred at 70  $^\circ\text{C}$  for 1 h. After cooling to room temperature, the precipitate was collected and washed with acetone (100 mL) to give 3-ethyl-4-oxo-5-[(phenylamino)methylidene]thiazoline-2-thione (45.0 g). This product was mixed with acetic anhydride (110 g, 1080 mmol) and triethylamine (0.18 g, 2 mmol), and the mixture was stirred at 110  $^\circ\text{C}$  for 30 min. The reaction mixture was concentrated to about one-half volume under reduced pressure. To this residue was added methanol (225 mL), and the mixture was stirred at 10  $^\circ\text{C}$  for 1 h. The precipitate formed was collected and washed with methanol (100 mL) to give 7 (37.0 g, 77.9%) as yellow crystals:  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  1.08 (t,  $J$  = 7.2 Hz, 3H), 2.02 (s, 3H), 3.95 (q,  $J$  = 7.2 Hz, 2H), 7.47–7.57 (m, 2H), 7.59–7.72 (m, 3H), 8.47 (s, 1H); MS (EI)  $m/z$  306. Anal. ( $\text{C}_{14}\text{H}_{14}\text{N}_2\text{O}_2\text{S}_2$ ) C, H, N, S.

**3-Ethyl-5-[2-(3-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)ethylidene]-4-oxothiazolidine-2-thione (8).** A mixture of 7 (29.8 g, 97 mmol), 3-ethyl-2-methylnaphtho[1,2-*d*]thiazolinium *p*-toluenesulfonate (38.8 g, 97 mmol), and acetic anhydride (14.2 g, 140 mmol) in acetonitrile (1000 mL) was stirred at 50  $^\circ\text{C}$  for 1 h. To this was added triethylamine (36.3 g, 359 mmol) at 50  $^\circ\text{C}$ , and the mixture was stirred for an additional 4 h at 60  $^\circ\text{C}$ . After cooling to 25  $^\circ\text{C}$ , the precipitate formed was collected and washed with acetonitrile (250 mL). The crude product thus obtained was suspended in methanol (750 mL), and the mixture was stirred under reflux for 1 h. After cooling to 25  $^\circ\text{C}$ , the precipitate was collected to give 8 (27.4 g, 70.7%) as purple-red crystals:  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  1.16 (t,  $J$  = 7.2 Hz, 3H), 1.59 (t,  $J$  = 7.2 Hz, 3H), 4.04 (q,  $J$  = 7.2 Hz, 2H), 4.66 (q,  $J$  = 7.2 Hz, 2H), 5.58 (d,  $J$  = 13.2 Hz, 1H), 7.54–7.69 (m, 3H), 7.85–7.93 (m, 2H), 8.08 (d,  $J$  = 9.0 Hz, 1H), 8.46 (d,  $J$  = 9.0 Hz, 1H); MS (HRMS) for  $\text{C}_{20}\text{H}_{18}\text{N}_2\text{O}_3\text{S}_3$  calcd 398.0581, found 398.0572. Anal. ( $\text{C}_{20}\text{H}_{18}\text{N}_2\text{O}_3\text{S}_3$ ) C, H, N: calcd, 24.13; found, 24.58.

**3-Ethyl-2-[[3-ethyl-5-[2-(3-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)ethylidene]-4-oxothiazolidin-2-ylidene]methyl]naphtho[1,2-*d*]thiazolium *p*-Toluenesulfonate (10).** A mixture of 8 (14.5 g, 36 mmol) and methyl *p*-toluenesulfonate (20.2 g, 109 mmol) in *N,N*-dimethylformamide (35 mL) and toluene (13 mL) was stirred at 115  $^\circ\text{C}$  for 6 h. To the mixture was added 3-ethyl-2-methylnaphtho[1,2-*d*]thiazolinium *p*-tolu-

enesulfonate (14.7 g, 36 mmol) in acetonitrile (1080 mL). To this was added triethylamine (11.0 g, 109 mmol) at 75  $^\circ\text{C}$ , and the resulting mixture was stirred at 75  $^\circ\text{C}$  for an additional 1 h and then cooled to 30  $^\circ\text{C}$ . The precipitate formed was collected and washed with acetonitrile (300 mL). The crude product thus obtained was suspended in methanol (720 mL), and the mixture was stirred under reflux for 1 h. After cooling to 25  $^\circ\text{C}$ , the precipitate was collected and washed with acetonitrile (300 mL). Compound 10 (20.4 g, 74.1%) was obtained as green crystals: UV-vis (MeOH)  $\lambda_{\text{max}}$  620.0 nm ( $\epsilon$  1.00  $\times 10^5$ );  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  1.28 (t,  $J$  = 7.2 Hz, 3H), 1.65–1.72 (m, 6H), 2.29 (s, 3H), 4.17 (q,  $J$  = 7.2 Hz, 2H), 4.72 (q,  $J$  = 7.2 Hz, 2H), 4.98 (q,  $J$  = 7.2 Hz, 2H), 6.02 (d,  $J$  = 12.9 Hz, 1H), 6.62 (s, 1H), 7.05–7.82 (m, 12H), 7.97 (d,  $J$  = 8.4 Hz, 1H), 8.03–8.12 (m, 2H), 8.30 (d,  $J$  = 8.4 Hz, 1H), 8.52 (d,  $J$  = 8.4 Hz, 1H); MS (FAB $^+$ , nitrobenzyl alcohol) for  $\text{C}_{34}\text{H}_{30}\text{N}_3\text{O}_3\text{S}_3$   $m/z$  592, (FAB $^-$ , nitrobenzyl alcohol) for  $\text{C}_{34}\text{H}_{30}\text{N}_3\text{O}_3\text{S}_3$   $m/z$  171; HRMS (FAB $^+$ ) for  $\text{C}_{34}\text{H}_{30}\text{N}_3\text{O}_3\text{S}_3$  calcd 592.1551, found 592.1561. Anal. ( $\text{C}_{41}\text{H}_{37}\text{N}_3\text{O}_4\text{S}_4 \cdot 2.7\text{H}_2\text{O}$ ) C, N, S; H: calcd, 5.26; found, 4.84.

**3-Ethyl-2-[[*N*-phenylamino]thiocarbonyl]methyl]benzothiazolium Iodide (12a).** To a suspension of sodium hydride (36.0 g, 1.5 mol) in tetrahydrofuran (1.1 L) was added 3-ethyl-2-methylbenzothiazolium iodide (11) (154.1 g, 0.5 mol) in a small portion at room temperature. To this was added dropwise a solution of phenyl thiocyanate (68.9 g, 0.5 mol) in tetrahydrofuran over a period of 1 h at 20–30  $^\circ\text{C}$ , and the resulting mixture was stirred under reflux for 30 min. The solvent was evaporated, and to the residue was added water (500 mL) dropwise under 10  $^\circ\text{C}$ . The precipitate formed was collected and washed with water (500 mL) to give 12a (131.3 g, 84.0%) as yellow crystals. Compound 12a was used for the next step without further purification.

**3-Ethyl-2-[(3-phenyl-4-oxothiazolidin-2-ylidene)methyl]benzothiazolium Bromide (13a).** A mixture of compound 12a (11.0 g, 35 mmol) and bromoacetic acid (11.0 g, 79 mmol) in 1-butanol (22 mL) was stirred at 100  $^\circ\text{C}$  for 10 min. After cooling to room temperature, to the mixture was added diethyl ether (100 mL). The precipitate formed was collected and washed with diethyl ether (50 mL) to give the crude product, which was recrystallized from methanol (75 mL) to give 13a (10.9 g, 73.0%) as brown crystals: UV-vis (MeOH)  $\lambda_{\text{max}}$  377.8 nm ( $\epsilon$  3.70  $\times 10^4$ );  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  1.13 (t,  $J$  = 7.2 Hz, 3H), 4.29 (q,  $J$  = 7.2 Hz, 2H), 4.58 (s, 2H), 5.97 (s, 1H), 7.52 (dd,  $J$  = 7.5, 1.5 Hz, 2H), 7.63–7.75 (m, 4H), 7.79 (t,  $J$  = 7.2 Hz, 1H), 8.11 (d,  $J$  = 7.9 Hz, 1H), 8.41 (d,  $J$  = 7.9 Hz, 1H); MS (FAB $^+$ , glycerine) for  $\text{C}_{19}\text{H}_{17}\text{N}_2\text{O}_2\text{S}_2$   $m/z$  353, (FAB $^-$ , glycerine) for Br  $m/z$  79, 81. Anal. ( $\text{C}_{19}\text{H}_{17}\text{BrN}_2\text{O}_2\text{S}_2 \cdot 1.1\text{H}_2\text{O}$ ) C, H, Br, N, S.

**3-Ethyl-2-[[*N*-ethylamino]thiocarbonyl]methyl]benzothiazolium Iodide (12b).** A solution of 3-ethyl-2-methylbenzothiazolium iodide (11) (36.0 g, 118 mmol) and triethylamine (4.4 g, 43 mmol) in pyridine (60 mL) was stirred at 110  $^\circ\text{C}$  for 20 min, and then the reaction mixture was poured into water (2000 mL). The precipitate formed was collected to give 12b (19.0 g, 60.9%) as purple crystals. Compound 12a was used for the next step without further purification.

**3-Ethyl-2-[(3-ethyl-4-oxothiazolidin-2-ylidene)methyl]benzothiazolium Bromide (13b).** A solution of compound 12b (6.0 g, 23 mmol) and bromoacetic acid (6.0 g, 43 mmol) in acetic acid (20 mL) was stirred at 90  $^\circ\text{C}$  for 5 min. After the mixture cooled to room temperature, diethyl ether (30 mL) was added. The precipitate formed was collected and washed with diethyl ether (20 mL) to give the crude product, which was recrystallized from methanol (350 mL) to give 13b (5.6 g, 77.1%) as yellow crystals: UV-vis (MeOH)  $\lambda_{\text{max}}$  390.4 nm ( $\epsilon$  4.20  $\times 10^4$ );  $^1\text{H-NMR}$  (DMSO- $d_6$ )  $\delta$  1.29 (t,  $J$  = 7.2 Hz, 3H), 1.39 (t,  $J$  = 7.2 Hz, 3H), 4.09 (q,  $J$  = 7.2 Hz, 2H), 4.45 (s, 2H), 4.86 (q,  $J$  = 7.2 Hz, 2H), 6.82 (s, 1H), 7.67 (t,  $J$  = 7.5 Hz, 1H), 7.82 (t,  $J$  = 7.5 Hz, 1H), 8.15 (d,  $J$  = 7.9 Hz, 1H), 8.39 (d,  $J$  = 7.9 Hz, 1H); MS (FAB $^+$ , glycerine) for  $\text{C}_{15}\text{H}_{17}\text{N}_2\text{O}_2\text{S}_2$   $m/z$  305, (FAB $^-$ , glycerine) for Br  $m/z$  79, 81. Anal. ( $\text{C}_{15}\text{H}_{17}\text{BrN}_2\text{O}_2\text{S}_2 \cdot 0.7\text{H}_2\text{O}$ ) C, H, Br, N, S.

**3-Ethyl-2-[(3-phenyl-5-[(*N*-phenylamino)methylidene]-4-oxothiazolidin-2-ylidene)methyl]benzothiazolium Bromide (14).** A mixture of 13a (13.0 g, 30 mmol) and 1,3-diaza-

ML) was stirred at 80 °C for 30 min. After cooling to room temperature, to this mixture was added diethyl ether (30 mL), and the precipitate formed was collected. The crude product thus obtained was suspended in 2-propanol (60 mL) and stirred at room temperature for 10 min. The precipitate was collected and washed with isopropyl alcohol (30 mL) to give 14 (18.0 g, quantitative) as yellow crystals: UV-vis (MeOH)  $\lambda_{\max}$  480.3 nm ( $\epsilon$  6.26  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.14 (t, *J* = 7.2 Hz, 3H), 4.26 (q, *J* = 7.2 Hz, 2H), 6.00 (s, 1H), 7.12–7.21 (m, 1H), 7.40–7.51 (m, 3H), 7.53–7.82 (m, 7H), 8.06 (d, *J* = 7.5 Hz, 1H), 8.36 (d, *J* = 7.5 Hz, 1H), 8.46 (t, *J* = 7.5 Hz, 1H), 11.01 (d, *J* = 13.2 Hz, 1H); MS (FAB<sup>+</sup>, glycerine) for C<sub>26</sub>H<sub>22</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub> *m/z* 456, (FAB<sup>+</sup>, nitrobenzyl alcohol) for Br *m/z* 79, 81. Anal. (C<sub>26</sub>H<sub>22</sub>BrN<sub>3</sub>O<sub>2</sub>S<sub>2</sub>·0.5H<sub>2</sub>O) C, H, Br, N, S.

**3-Ethyl-2-[[3-phenyl-5-[(*N*-phenylamino)-2-propenylidene]-4-oxothiazolidin-2-ylidene]methyl]benzothiazolium Iodide (15).** A mixture of 13a (8.7 g, 20 mmol), 1,5-diaza-1,5-diphenyl-1,3-pentadiene hydrochloride salt (25.9 g, 100 mmol), and triethylamine (13.0 g, 128 mmol) in methanol (170 mL) was stirred at 50 °C for 20 min. After the mixture cooled to room temperature, a solution of sodium iodide (9.0 g, 60 mmol) in methanol (45 mL) was added. The precipitate formed was collected to give the crude product, which was suspended in ethanol (160 mL) and stirred at 50 °C for 20 min. The precipitate was collected and washed with ethanol (30 mL) to give 14 (9.8 g, 80.3%) as dark-green crystals: UV-vis (MeOH)  $\lambda_{\max}$  547.4 nm ( $\epsilon$  7.46  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.13 (t, *J* = 7.2 Hz, 3H), 4.22 (q, *J* = 7.2 Hz, 2H), 5.89 (t, *J* = 12.0 Hz, 1H), 6.04 (s, 1H), 7.07 (t, *J* = 7.5 Hz, 1H), 7.19 (d, *J* = 8.1 Hz, 2H), 7.36 (t, *J* = 7.5 Hz, 2H), 7.56–7.78 (m, 7H), 7.95–8.06 (m, 2H), 8.30–8.45 (m, 2H), 10.67 (d, *J* = 12.6 Hz, 1H); MS (FAB<sup>+</sup>, glycerine) for C<sub>28</sub>H<sub>24</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub> *m/z* 482, (FAB<sup>+</sup>, nitrobenzyl alcohol) for I *m/z* 127. Anal. (C<sub>28</sub>H<sub>24</sub>IN<sub>3</sub>O<sub>2</sub>S<sub>2</sub>·1.0H<sub>2</sub>O) C, H, I, N, S.

**4-[2-(3-Ethylbenzoxazolin-2-ylidene)ethylidene]-2-(ethylthio)-5-oxothiazoline (18).** To a solution of carbodithioate 16 (17.9 g, 0.10 mol) in acetic anhydride (80 mL) was added triethyl orthoformate (21.3 g, 0.14 mol), and the mixture was refluxed 1.5 h. After the acetic anhydride was evaporated, to the residue were added 3-ethyl-2-methylbenzoxazolinium iodide (28.9 g, 0.10 mol) in ethanol (80 mL) and then triethylamine (18.6 g, 0.18 mol). The resulting mixture was refluxed for 15 min and then cooled to 10 °C. The precipitate formed was collected to give merocyanine dye intermediate 18 (25.9 g, 78.0%). Compound 18 was used for the next step without further purification.

**4-[2-(3-Ethylbenzoxazolin-2-ylidene)ethylidene]-2-(ethylthio)-3-methyl-5-oxothiazolinium *p*-Toluenesulfonate (19).** A mixture of compound 18 (24.9 g, 75 mmol) and methyl *p*-toluenesulfonate (44.7 g, 240 mmol) was stirred at 130 °C for 1 h. After cooling to 40 °C, to the reaction mixture were added ethanol (30 mL) and then diethyl ether (100 mL). The precipitate was collected to give 19 (30.1 g, 77.4%): <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.41 (t, *J* = 7.2 Hz, 6H), 2.29 (s, 3H), 3.92 (s, 3H), 4.35 (q, *J* = 7.2 Hz, 2H), 7.03–7.13 (m, 3H), 7.47 (d, *J* = 8.9 Hz, 2H), 7.51–7.61 (m, 2H), 7.81–7.92 (m, 2H), 7.98 (d, *J* = 13.8 Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>17</sub>H<sub>19</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub> *m/z* 347, (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>7</sub>H<sub>7</sub>O<sub>3</sub>S *m/z* 171; HRMS (FAB<sup>+</sup>) for C<sub>17</sub>H<sub>19</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub> calcd 347.0888, found 347.0886.

**3-Ethyl-2-[[3-methyl-4-[2-(3-ethylbenzoxazolin-2-ylidene)ethylidene]-5-oxothiazolidin-2-ylidenemethyl]benzothiazolium Iodide (20).** To a mixture of 19 (10.4 g, 20 mmol) and 3-ethyl-2-methylbenzothiazolium iodide (6.1 g, 20 mmol) in ethanol (65 mL) was added triethylamine (2.6 g, 26 mmol) at room temperature, and the reaction mixture was refluxed for 30 min. After cooling to room temperature, the precipitate was collected and washed with ethanol (30 mL) to yield 20 (7.46 g, 63.3%): UV-vis (MeOH)  $\lambda_{\max}$  574.1 nm ( $\epsilon$  2.09  $\times$  10<sup>5</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.31 (t, *J* = 7.2 Hz, 3H), 1.34 (t, *J* = 7.2 Hz, 3H), 3.83 (s, 3H), 4.15 (q, *J* = 7.2 Hz, 2H), 4.52 (q, *J* = 7.2 Hz, 2H), 6.46 (s, 1H), 6.78 (d, *J* = 13.2 Hz, 1H), 7.29–7.55 (m, 4H), 7.61–7.69 (m, 4H), 7.97 (d, *J* = 7.0 Hz, 1H); MS (FAB<sup>+</sup>, glycerine) for C<sub>25</sub>H<sub>24</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub> *m/z* 462, (FAB<sup>+</sup>, glycerine) for I *m/z* 127. Anal. (C<sub>25</sub>H<sub>24</sub>IN<sub>3</sub>O<sub>2</sub>S<sub>2</sub>·1H<sub>2</sub>O) C, H, I, N, S.

**3-Ethyl-2-[[3-(3-ethylbenzothiazolin-2-ylidene)-1-propenyl]benzothiazolium Iodide (21).** This compound was synthesized from 3-ethylbenzothiazolium iodide and triethyl orthoformate in pyridine according to the reported method:<sup>10</sup> UV-vis (MeOH)  $\lambda_{\max}$  555.8 nm ( $\epsilon$  1.56  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.35 (t, *J* = 7.1 Hz, 6H), 4.38 (q, *J* = 7.1 Hz, 4H), 6.67 (d, *J* = 12.6 Hz, 2H), 7.41 (t, *J* = 7.8 Hz, 2H), 7.58 (t, *J* = 7.8 Hz, 2H), 7.72–7.83 (m, 3H), 8.01 (d, *J* = 7.8 Hz, 2H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>21</sub>H<sub>21</sub>N<sub>2</sub>S<sub>2</sub> *m/z* 365, (FAB<sup>+</sup>, nitrobenzyl alcohol) for I *m/z* 127. Anal. (C<sub>21</sub>H<sub>21</sub>IN<sub>2</sub>S<sub>2</sub>·1.1H<sub>2</sub>O) C, H, I, N, S.

**3-Ethyl-2-[[3-chloro-5-(3-ethylnaphtho[1,2-*d*]thiazolin-2-ylidene)-1,3-pentadienyl]naphtho[1,2-*d*]thiazolium *p*-Toluenesulfonate (22).** This compound was synthesized from 3-ethylbenzothiazolium iodide and 3-chloro-1,5-diaza-1,5-diphenyl-1,3-pentadiene in pyridine in a manner analogous to the preparation of 21: UV-vis (MeOH)  $\lambda_{\max}$  681.0 nm ( $\epsilon$  2.42  $\times$  10<sup>5</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.66 (t, *J* = 7.2 Hz, 6H), 2.29 (s, 3H), 4.73 (q, *J* = 7.2 Hz, 4H), 6.32 (d, *J* = 12.9 Hz, 2H), 7.11 (d, *J* = 8.1 Hz, 2H), 7.48 (d, *J* = 8.1 Hz, 2H), 7.55 (t, *J* = 7.5 Hz, 2H), 7.70 (t, *J* = 7.5 Hz, 2H), 7.90 (d, *J* = 12.9 Hz, 2H), 7.97 (d, *J* = 8.7 Hz, 2H), 8.03–8.11 (m, 4H), 8.42 (d, *J* = 8.7 Hz, 2H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>31</sub>H<sub>26</sub>ClN<sub>2</sub>S<sub>2</sub> *m/z* 525, (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>7</sub>H<sub>7</sub>O<sub>3</sub>S *m/z* 171. Anal. (C<sub>38</sub>H<sub>33</sub>ClN<sub>2</sub>O<sub>3</sub>S<sub>3</sub>·1H<sub>2</sub>O) C, H, Cl, N, S.

**2-[[5-Cyclohexylidene-3-ethyl-4-oxothiazolidin-2-ylidenemethyl]-3-ethylbenzothiazolium *p*-Toluenesulfonate (23).** This compound was synthesized from 5-cyclohexylidene-3-ethyl-4-oxothiazolidine-2-thione and 3-ethyl-2-methylbenzothiazolium *p*-toluenesulfonate in a manner analogous to the preparation of rhodacyanine dye 5: UV-vis (MeOH)  $\lambda_{\max}$  425.8 nm ( $\epsilon$  5.97  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>)  $\delta$  1.29 (t, *J* = 7.1 Hz, 3H), 1.52 (t, *J* = 7.1 Hz, 3H), 1.68–1.80 (m, 2H), 1.92–2.10 (m, 4H), 2.31 (s, 3H), 2.47 (t, *J* = 6.6 Hz, 2H), 3.22 (t, *J* = 6.6 Hz, 2H), 4.33 (q, *J* = 7.1 Hz, 2H), 5.06 (q, *J* = 7.1 Hz, 2H), 7.10 (d, *J* = 7.8 Hz, 2H), 7.19 (s, 1H), 7.52–7.58 (m, 1H), 7.68 (d, *J* = 7.8 Hz, 1H), 7.77 (d, *J* = 8.1 Hz, 1H), 8.02 (d, *J* = 7.8 Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>21</sub>H<sub>25</sub>N<sub>2</sub>O<sub>2</sub>S<sub>2</sub> *m/z* 385, (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>7</sub>H<sub>7</sub>O<sub>3</sub>S *m/z* 171. Anal. (C<sub>28</sub>H<sub>32</sub>N<sub>2</sub>O<sub>4</sub>S<sub>3</sub>·1H<sub>2</sub>O) C, H, N, S.

**3-Ethyl-2-[[3-phenyl-5-(3-methylbenzothiazolin-2-ylidene)-4-oxothiazolidin-2-ylidenemethyl]benzothiazolium *p*-Toluenesulfonate (24).** This compound was synthesized from 2-(methylthio)benzothiazole and 4-oxo-3-phenylthiazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 5: UV-vis (MeOH)  $\lambda_{\max}$  501.0 nm ( $\epsilon$  6.83  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.14 (t, *J* = 7.1 Hz, 3H), 2.28 (s, 3H), 4.20 (q, *J* = 7.1 Hz, 2H), 4.30 (s, 3H), 5.93 (s, 1H), 7.10 (d, *J* = 7.8 Hz, 2H), 7.39 (t, *J* = 8.1 Hz, 1H), 7.48 (d, *J* = 7.8 Hz, 2H), 7.51–7.62 (m, 4H), 7.66–7.76 (m, 4H), 7.82 (d, *J* = 7.8 Hz, 1H), 7.94 (d, *J* = 7.8 Hz, 1H), 7.98 (d, *J* = 7.8 Hz, 1H), 8.28 (d, *J* = 7.6 Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>27</sub>H<sub>22</sub>N<sub>3</sub>O<sub>3</sub>S<sub>3</sub> *m/z* 500, (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>7</sub>H<sub>7</sub>O<sub>3</sub>S *m/z* 171; HRMS (FAB<sup>+</sup>) for C<sub>27</sub>H<sub>22</sub>N<sub>3</sub>O<sub>3</sub>S<sub>3</sub> calcd 500.0925, found 500.0908.

**3-Ethyl-2-[[3-phenyl-5-[2-(3-ethylbenzothiazolin-2-ylidene)ethylidene]-4-oxothiazolidin-2-ylidenemethyl]benzothiazolium Iodide (25).** This compound was synthesized from 3-ethyl-2-methylbenzothiazolium *p*-toluenesulfonate and 4-oxo-3-phenylthiazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 10: UV-vis (MeOH)  $\lambda_{\max}$  595.4 nm ( $\epsilon$  1.07  $\times$  10<sup>5</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.13 (t, *J* = 7.1 Hz, 3H), 1.33 (t, *J* = 7.1 Hz, 3H), 4.19 (q, *J* = 7.1 Hz, 2H), 4.38 (q, *J* = 7.1 Hz, 2H), 5.99 (s, 1H), 6.18 (d, *J* = 13.2 Hz, 1H), 7.32 (t, *J* = 7.2 Hz, 1H), 7.45–7.95 (m, 12H), 8.29 (d, *J* = 7.2 Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>30</sub>H<sub>26</sub>N<sub>3</sub>O<sub>1</sub>S<sub>3</sub> *m/z* 540, (FAB<sup>+</sup>, nitrobenzyl alcohol) for I *m/z* 127. Anal. (C<sub>30</sub>H<sub>26</sub>IN<sub>3</sub>O<sub>1</sub>S<sub>3</sub>·0.3H<sub>2</sub>O) C, H, I, N, S.

**2-[[1,3-Diethyl-5-(3-methylbenzothiazolin-2-ylidene)-4-oxoimidazolidin-2-ylidenemethyl]-3-ethylbenzothiazolium Iodide (26).** This compound was synthesized from 2-(methylthio)benzothiazole and 1,3-diethyl-4-oxoimidazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 5: UV-vis (MeOH)  $\lambda_{\max}$  481.0 nm ( $\epsilon$  4.83  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.01 (t, *J* = 6.9 Hz, 3H), 1.23 (t, *J* = 7.1 Hz, 3H), 1.35 (t, *J* = 7.1 Hz, 3H), 3.95 (q, *J* = 7.2 Hz,

2H), 4.05 (s, 3H), 4.08 (q,  $J = 7.2$  Hz, 2H), 4.42 (q,  $J = 7.2$  Hz, 2H), 5.88 (s, 1H), 7.36 (t,  $J = 8.0$  Hz, 1H), 7.44 (t,  $J = 8.0$  Hz, 1H), 7.54 (t,  $J = 8.0$  Hz, 1H), 7.49–7.75 (m, 3H), 7.94 (d,  $J = 7.5$  Hz, 1H), 8.04 (d,  $J = 7.5$  Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>23</sub>H<sub>27</sub>N<sub>4</sub>O<sub>5</sub>  $m/z$  463, (FAB<sup>+</sup>, nitrobenzyl alcohol) for  $m/z$  127. Anal. (C<sub>23</sub>H<sub>27</sub>N<sub>4</sub>O<sub>5</sub>) C, H, I, N, S.

**3-Ethyl-2-[[3-ethyl-5-[[2-(3-ethyl-2-methyl-1,2-d]thiazolin-2-ylidene)ethylidene]-4-oxothiazolidin-2-ylidene]methyl]naphtho[2,1-d]thiazolium *p*-Toluenesulfonate (27).** This compound was synthesized from 3-ethyl-2-methyl[1,2-d]naphthothiazolium *p*-toluenesulfonate and 3-ethyl-4-oxothiazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 10: UV-vis (MeOH)  $\lambda_{\max}$  622.9 nm ( $\epsilon$  1.07  $\times$  10<sup>5</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.27 (t,  $J = 6.9$  Hz, 3H), 1.38 (t,  $J = 7.0$  Hz, 3H), 1.72 (t,  $J = 6.9$  Hz, 3H), 2.30 (s, 3H), 4.08 (q,  $J = 6.9$  Hz, 2H), 4.52–4.70 (m, 4H), 5.94 (d,  $J = 13.2$  Hz, 1H), 6.39 (s, 1H), 6.77–6.88 (m, 2H), 7.14 (d,  $J = 8.1$  Hz, 2H), 7.35–7.748 (m, 2H), 7.50–7.67 (m, 6H), 7.78–7.95 (m, 5H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>34</sub>H<sub>30</sub>N<sub>3</sub>O<sub>5</sub>  $m/z$  592, (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>7</sub>H<sub>7</sub>O<sub>3</sub>S  $m/z$  171; HRMS (FAB<sup>+</sup>) for C<sub>34</sub>H<sub>30</sub>N<sub>3</sub>O<sub>5</sub> calcd 592.1551, found, 592.1558.

**2-[[1,3-Diethyl-5-[[2-(3-ethylnaphtho[2,1-d]thiazolin-2-ylidene)ethylidene]-4-oxoimidazolidin-2-ylidenemethyl]-3-ethylnaphtho[2,1-d]thiazolium Iodide (28).** This compound was synthesized from 3-ethyl-2-methyl[1,2-d]naphthothiazolium *p*-toluenesulfonate and 1,3-diethyl-4-oxoimidazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 10: UV-vis (MeOH)  $\lambda_{\max}$  606.3 nm ( $\epsilon$  1.04  $\times$  10<sup>5</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.23–1.30 (m, 6H), 1.38 (t,  $J = 7.2$  Hz, 3H), 1.74 (t,  $J = 7.2$  Hz, 3H), 3.95 (q,  $J = 7.2$  Hz, 2H), 4.30 (q,  $J = 7.2$  Hz, 2H), 4.41 (q,  $J = 7.2$  Hz, 2H), 4.69 (t,  $J = 7.2$  Hz, 2H), 5.77 (s, 1H), 7.50–7.58 (m, 3H), 7.62–7.84 (m, 5H), 7.95–8.18 (m, 5H), 8.47 (1H,  $J = 8.5$  Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>36</sub>H<sub>35</sub>N<sub>4</sub>O<sub>5</sub>  $m/z$  603, (FAB<sup>+</sup>, nitrobenzyl alcohol) for  $m/z$  127. Anal. (C<sub>36</sub>H<sub>35</sub>N<sub>4</sub>O<sub>5</sub>·1.5H<sub>2</sub>O) C, H, I, N, S.

**3-Ethyl-2-[[3-methyl-5-[[2-(3-ethylbenzoxazolin-2-ylidene)ethylidene]-4-oxothiazolidin-2-ylidene]methyl]benzothiazolium Iodide (29).** This compound was synthesized from 3-ethyl-2-methylbenzoxazolinium *p*-toluenesulfonate and 3-methyl-4-oxothiazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 10: UV-vis (MeOH)  $\lambda_{\max}$  564.3 nm ( $\epsilon$  8.76  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.33–1.43 (m, 6H), 3.60 (s, 3H), 4.23 (q,  $J = 7.1$  Hz, 2H), 4.72 (q,  $J = 7.1$  Hz, 2H), 5.62 (d,  $J = 13.2$  Hz, 1H), 6.70 (s, 1H), 7.28–7.42 (m, 2H), 7.53–7.75 (m, 4H), 7.96–8.08 (m, 2H), 8.24 (d,  $J = 7.2$  Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>25</sub>H<sub>24</sub>N<sub>3</sub>O<sub>5</sub>  $m/z$  462, (FAB<sup>+</sup>, nitrobenzyl alcohol) for  $m/z$  127; HRMS (FAB<sup>+</sup>) for C<sub>25</sub>H<sub>24</sub>N<sub>3</sub>O<sub>5</sub> calcd 462.1310, found 462.1309. Anal. (C<sub>25</sub>H<sub>24</sub>N<sub>3</sub>O<sub>5</sub>·1.75H<sub>2</sub>O) C, I, N, S; H: calcd, 4.46; found, 3.92.

**3-Ethyl-2-[[5-[[2-(3-ethylbenzoxazolin-2-ylidene)ethylidene]-4-oxo-1,3-dithiolan-2-ylidene]methyl]benzothiazolium iodide (30).** This compound was synthesized according to the reported method<sup>16</sup> from 3-ethyl-2-methylbenzothiazolinium iodide and carbon disulfide: UV-vis (MeOH)  $\lambda_{\max}$  586.8 nm ( $\epsilon$  6.54  $\times$  10<sup>4</sup>); NMR studies (COSY and ROESY spectra (data are not shown)) revealed that compound 30 was a mixture (1:1) of geometrical isomers at the merocyanine moiety, <sup>1</sup>H-NMR (CDCl<sub>3</sub>:DMSO-*d*<sub>6</sub> = 3.5:1.5) (one isomer)  $\delta$  1.47–1.55 (m, 6H), 4.33 (q,  $J = 7.2$  Hz, 2H), 4.71–4.77 (m, 2H), 5.54 (d,  $J = 13.5$  Hz, 1H), 7.38 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.43 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.49 (d,  $J = 7.6$  Hz, 1H), 7.58 (d,  $J = 7.6$  Hz, 1H), 7.68 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.83 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.84 (s, 1H), 8.00 (d,  $J = 7.6$  Hz, 1H), 8.17 (d,  $J = 13.5$  Hz, 1H), 8.25 (d,  $J = 7.6$  Hz, 1H), (another isomer)  $\delta$  1.47–1.55 (m, 6H), 4.25 (q,  $J = 7.2$  Hz, 2H), 4.71–4.77 (m, 2H), 5.39 (d,  $J = 13.5$  Hz, 1H), 7.38 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.43 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.49 (d,  $J = 7.6$  Hz, 1H), 7.58 (d,  $J = 7.6$  Hz, 1H), 7.64 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.67 (s, 1H), 7.78 (dd,  $J = 7.6, 7.6$  Hz, 1H), 7.95 (d,  $J = 7.6$  Hz, 1H), 8.10 (d,  $J = 13.5$  Hz, 1H), 8.20 (d,  $J = 7.6$  Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>24</sub>H<sub>21</sub>N<sub>2</sub>O<sub>2</sub>S<sub>3</sub>  $m/z$  465, (FAB<sup>+</sup>, nitrobenzyl alcohol) for  $m/z$  127; HRMS (FAB<sup>+</sup>) for C<sub>24</sub>H<sub>21</sub>N<sub>2</sub>O<sub>2</sub>S<sub>3</sub> calcd 465.0765, found 465.0765. Anal. (C<sub>24</sub>H<sub>21</sub>N<sub>2</sub>O<sub>2</sub>S<sub>3</sub>·1.5H<sub>2</sub>O) C, H, N, S; I: calcd, 20.48; found, 19.85.

**3-Ethyl-2-[[3-ethyl-5-[[3-methylbenzothiazolin-2-ylidene)-**

**4-oxothiazolidin-2-ylidene]methyl]benzothiazolium Acetate (31).** A solution of 5 (4.2 g, 7 mmol) in a 0.1 M acetic acid solution (300 mL) of chloroform/methanol (1/2, v/v) was passed through the basic anion-exchange resin (about 120 mL, Diaion WA-21, acetate form), and the resin was washed with a 0.1 M acetic acid solution (200 mL) of methanol. The eluent was concentrated to about 50 mL, to which was added ethyl acetate (300 mL). The precipitate formed was collected and washed with ethyl acetate (50 mL) to give 31 (2.8 g, 67.4%) as orange crystals: UV-vis (MeOH)  $\lambda_{\max}$  501.2 nm ( $\epsilon$  6.88  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.26 (t,  $J = 7.1$  Hz, 3H), 1.37 (t,  $J = 7.1$  Hz, 3H), 1.68 (s, 3H), 4.21 (s, 4.5H), 4.28 (q,  $J = 7.2$  Hz, 2H), 4.70 (q,  $J = 7.2$  Hz, 2H), 6.67 (s, 1H), 7.33 (t,  $J = 7.7$  Hz, 1H), 7.50 (q,  $J = 7.8$  Hz, 2H), 7.71 (q,  $J = 8.5$  Hz, 2H), 7.94 (d,  $J = 8.1$  Hz, 2H), 8.24 (d,  $J = 8.1$  Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>22</sub>H<sub>22</sub>N<sub>3</sub>O<sub>5</sub>  $m/z$  452, (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>22</sub>H<sub>22</sub>N<sub>3</sub>O<sub>5</sub>  $m/z$  59; HRMS (FAB<sup>+</sup>) for C<sub>22</sub>H<sub>22</sub>N<sub>3</sub>O<sub>5</sub> calcd 452.0925, found 452.0891. Anal. (C<sub>22</sub>H<sub>22</sub>N<sub>3</sub>O<sub>5</sub>·2.85H<sub>2</sub>O·0.5C<sub>2</sub>H<sub>4</sub>O<sub>2</sub>) C, S; H: calcd, 5.56; found, 4.98. N: calcd, 7.09; found, 6.43.

**2-[[3-Cyclohexyl-5-[[3-methylbenzothiazolin-2-ylidene)-4-oxothiazolidin-2-ylidene]methyl]-3-ethylbenzoxazolinium Chloride (32).** This compound was synthesized from 2-(methylthio)benzothiazole and 4-oxo-3-cyclohexylthiazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 5: UV-vis (MeOH)  $\lambda_{\max}$  488.1 nm ( $\epsilon$  6.73  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>)  $\delta$  1.24–1.90 (m, 10H), 4.18 (s, 3H), 4.50 (q,  $J = 7.0$  Hz, 2H), 4.60 (brs, 1H), 6.36 (s, 1H), 7.48 (dd,  $J = 7.8, 7.8$  Hz, 1H), 7.48–7.61 (m, 3H), 7.79 (dd,  $J = 8.0, 8.0$  Hz, 2H), 7.98 (dd,  $J = 7.8, 7.8$  Hz, 2H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>27</sub>H<sub>28</sub>N<sub>3</sub>O<sub>2</sub>S<sub>2</sub>  $m/z$  490, (FAB<sup>+</sup>, nitrobenzyl alcohol) for Cl  $m/z$  35. Anal. (C<sub>27</sub>H<sub>28</sub>ClN<sub>3</sub>O<sub>2</sub>S<sub>2</sub>·2.5H<sub>2</sub>O) C, H, Cl, N, O, S.

**3-Ethyl-2-[[3-ethyl-5-[[2-(3-ethyl-5-(trifluoromethyl)benzothiazolin-2-ylidene)ethylidene]-4-oxothiazolidin-2-ylidene]methyl]-4-methylthiazolium Iodide (33).** This compound was synthesized from 3-ethyl-2-methyl-5-trifluorobenzothiazolium *p*-toluenesulfonate and 3-methyl-4-oxothiazolidine-2-thione in a manner analogous to the preparation of rhodacyanine dye 10: UV-vis (MeOH)  $\lambda_{\max}$  559.2 nm ( $\epsilon$  8.39  $\times$  10<sup>4</sup>); <sup>1</sup>H-NMR (CD<sub>3</sub>OD)  $\delta$  1.32 (t,  $J = 7.1$  Hz, 3H), 1.40 (t,  $J = 7.1$  Hz, 3H), 1.45 (t,  $J = 7.1$  Hz, 3H), 2.53 (s, 3H), 4.14 (q,  $J = 7.1$  Hz, 2H), 4.31 (q,  $J = 7.1$  Hz, 2H), 4.46 (q,  $J = 7.2$  Hz, 2H), 5.87 (d,  $J = 12.5$  Hz, 1H), 6.59 (s, 1H), 7.49 (s, 1H), 7.51 (d,  $J = 8.9$  Hz, 1H), 7.65 (s, 1H), 7.84 (d,  $J = 12.5$  Hz, 1H), 7.85 (d,  $J = 8.9$  Hz, 1H); MS (FAB<sup>+</sup>, nitrobenzyl alcohol) for C<sub>24</sub>H<sub>25</sub>F<sub>3</sub>N<sub>3</sub>O<sub>5</sub>  $m/z$  524, (FAB<sup>+</sup>, nitrobenzyl alcohol) for  $m/z$  127; HRMS (FAB<sup>+</sup>) for C<sub>24</sub>H<sub>25</sub>F<sub>3</sub>N<sub>3</sub>O<sub>5</sub> calcd 524.1112, found 524.1108. Anal. (C<sub>24</sub>H<sub>25</sub>F<sub>3</sub>N<sub>3</sub>O<sub>5</sub>·2H<sub>2</sub>O) H, F, N, S; C: calcd, 4.25; found, 3.69. I: calcd, 18.46; found, 17.85.

**X-ray Structure Analysis of 32 and 33.** Compounds 32 and 33 were crystallized from ethanol and acetonitrile solutions, respectively. X-ray diffraction data were measured by a Rigaku AFC-5R instrument using Cu K $\alpha$  radiation and a graphite monochromometer. Structures were determined by direct-method SHELXS86 and successive Fourier syntheses and refined by a full-matrix least-squares method. Full crystallographic details are available as Supporting Information.

**In Vitro Clonogenic Assay.** CX-1 and CV-1 cell lines were grown in a 50:50 mix of Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% calf serum (HyClone Laboratories Inc., Logan, UT) and antibiotics at 37 °C under 5% CO<sub>2</sub>, 95% air, and 100% humidity. CV-1 (normal African green monkey kidney) was obtained from the American Type Culture Collection (Rockville, MD), and CX-1 (human colon carcinoma) from Dr. M. Wolpert (National Cancer Institute).

For the clonogenic assay, cells were seeded at 1500 cells/wells for CX-1 and 1000 cells/wells for CV-1 in 96-well plates (Becton Dickinson Labware, Lincoln Park, NJ). The assay was performed in duplicate. The drugs were first dissolved in dimethyl sulfoxide, to prepare 10 mg/mL stock solutions. The final drug solution was made by mixing 100  $\mu$ L of this stock solution with 10 mL of 5% CS DME media solution. On the

following day, cells were treated with compounds at varied concentrations and cultured precisely for 24 h in the media. After rinsing, cells were incubated in drug-free medium for 2 weeks. Colonies were stained with 2% crystal violet in 70% ethanol and counted by an automated colony counter (Artek counter model 880, Dynatech Laboratories Inc., Chantilly, VA).

**In Vivo Human Tumor Xenografts in Nude Mice.** Male Swiss nu/nu mice (about 5 weeks old) were obtained from Taconic Farm, Inc. (Germantown, NY). Group housing (5/cage) was provided in polycarbonate cages with a wire top and filters. Mice were allowed to acclimate for 1 week prior to experiments. Only normal, healthy mice were used. Human melanoma LOX cells used in this model were first grown *in vivo* in nude mice. On the day of ip implantation, tumors were excised and a single cell suspension was prepared. RBCs were lysed by ammonium chloride. Each mouse received  $2 \times 10^6$  LOX cells (trypan blue-negative) in 0.2 mL of PBS by ip injection. Test compounds (0.2 mL/20 g of mouse body weight, 45% encapsin HPB (hydroxypropyl  $\beta$ -cyclodextrin; American Maise-Products Co.) in water) were administered ip for LOX tumor-bearing mice with appropriately determined doses and schedules. Evaluation against human ovarian carcinoma OVCARIII cells and human colon carcinoma CX-1 cells was performed in a manner analogous to the LOX ip tumor model.

**Acknowledgment.** We thank Dr. Hiroshi Kitaguchi for helpful discussions and Mr. Hiroo Fukunaga for advice about a molecular orbital calculation. We also thank Professor George Sheldrick of Institut für Anorganische Chemie der Universität for providing us with SHELXS86 for the X-ray structure analysis.

**Supporting Information Available:** X-ray crystallographic data for compounds 32 and 33 (21 pages). Ordering information is given on any current masthead page.

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## **EXHIBIT C**

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14. Supported in part by EPA grants R806569 and EPA-OTS subcontract T-6419 (7197)-033. We thank Peter Cornell, Mary Lou Krumme, and David Reynolds for technical assistance. Published as article No. 10158 of the Michigan Agricultural Experiment Station.

E. SHANKAR NARAYAN Ph.D.

## Rhodamine-123 Selectively Reduces Clonal Growth of Carcinoma Cells in vitro

**Abstract.** Rhodamine-123, a cationic laser dye, markedly reduced the clonal growth of carcinoma cells but had little effect on nontumorigenic epithelial cells in vitro. This selective inhibitory effect of Rhodamine-123 on some carcinomas is unusual since known anticancer drugs, such as arabinosyl cytosine and methotrexate, have not been shown to exhibit such selectivity in vitro.

The fluorescent dye Rhodamine-123 (Rh-123) selectively accumulates in the mitochondria of living cells (1). The specific accumulation of this dye appears to depend on its cationic and permeant properties and on the high electric potential (inside negative) across the mitochondrial membrane (2, 3). Although all living cells we have examined thus far accumulate Rh-123 in their mitochondria, cell types differ in their ability to retain Rh-123 in dye-free medium (3, 4). We found that most carcinoma cells retain Rh-123 for 2 to 5 days when they are incubated in dye-free medium, whereas nontumorigenic epithelial cells and tumorigenic or nontumorigenic cells of fibroblastic, neural, or hematopoietic origin release the dye within 1 to 16 hours (4). It may be possible to exploit the difference in Rh-123 retention between carcinoma cells and other cell types for cancer chemotherapy (5). In the study described herein, we compared the effects of Rh-123 treatment on the clonal growth of carcinoma cells and nontumorigenic epithelial cells in vitro.

For these experiments we used MB 49 cells, a mouse bladder epithelial line transformed by 7,12-dimethylbenz[*a*]anthracene (DMBA) (6). These cells are highly tumorigenic and retain a significant amount of Rh-123 in their mitochondria for 4 days when they are incubated

cultures of normal bladder epithelial cells lose Rh-123 fluorescence within 2 hours (4). To determine whether this difference in retention results in greater inhibition of clonal growth of MB 49 cells than of normal mouse bladder epithelial cells, we treated these cells grown in vitro with Rh-123 and assayed their colony-forming ability. Exposure to Rh-123 (10  $\mu$ g/ml) for 24 hours had a minimal effect on the colony-forming units (CFU) of normal mouse bladder epithelial cells (92 percent of control) (Fig. 1A), but markedly reduced the CFU of MB 49 cells (4 percent of control). The effect of Rh-123 on MB 49 cells depended on the concentration and duration of exposure. Even 6 hours of exposure to 10  $\mu$ g of Rh-123 per milliliter reduced the CFU to 45 percent of control, whereas such treatment had no significant effect on the CFU of normal bladder epithelial cells.

We then compared the reductions in CFU of EJ cells (a human bladder carcinoma line), MB 49 cells, and normal mouse bladder epithelial cells that had been exposed for 24 hours or continuously to different concentrations of Rh-123 during the 2-week period of clonal cell growth (Fig. 1, B and C). Continuous exposure of normal mouse bladder epithelial cells to Rh-123 (10  $\mu$ g/ml) had only a small effect on CFU. However, both EJ and MB 49 cells were susceptible to the inhibitory effects of Rh-123. Colony formation in these cells was reduced to 50 percent of control after 24 hours of exposure to 2 to 5  $\mu$ g of Rh-123

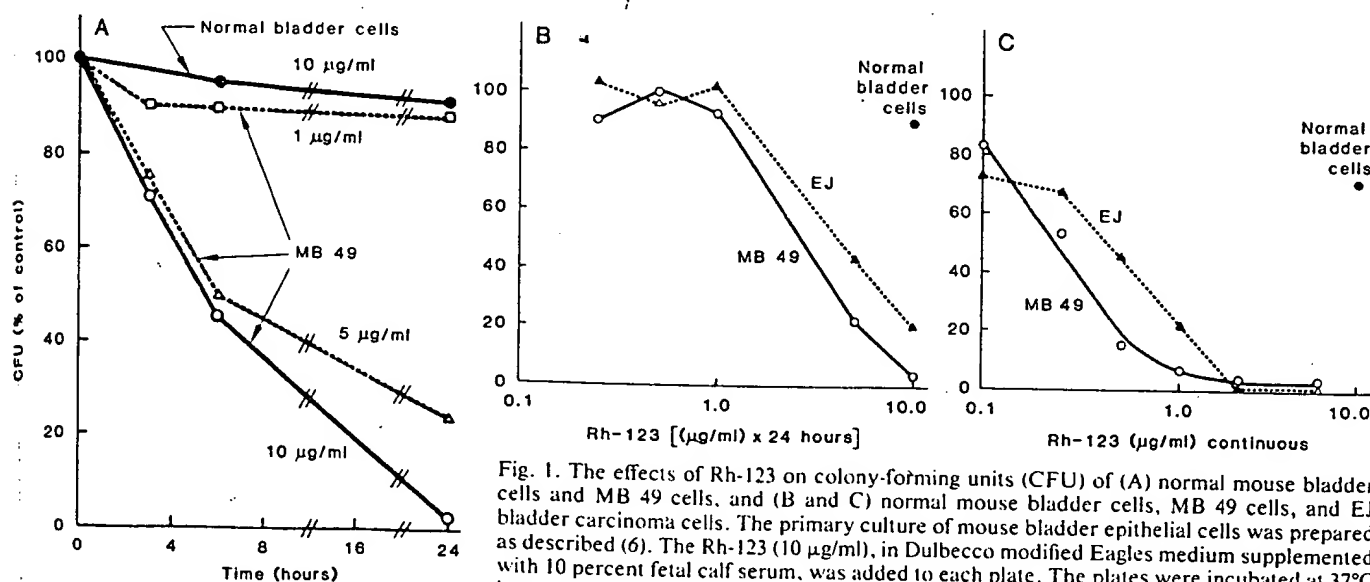


Fig. 1. The effects of Rh-123 on colony-forming units (CFU) of (A) normal mouse bladder cells and MB 49 cells, and (B and C) normal mouse bladder cells, MB 49 cells, and EJ bladder carcinoma cells. The primary culture of mouse bladder epithelial cells was prepared as described (6). The Rh-123 (10  $\mu$ g/ml), in Dulbecco modified Eagles medium supplemented with 10 percent fetal calf serum, was added to each plate. The plates were incubated at 37°C in 5 percent CO<sub>2</sub> for various times before the cells were washed and reincubated in rhodamine-free medium. After 2 weeks, the numbers of colonies were counted and the results were expressed as percentages of control, with control plates normalized to 100 percent CFU. The standard error for triplicate samples was 3 to 5 percent. The MB 49 cells (a mouse bladder epithelial cell line transformed with DMBA) and EJ cells (human bladder carcinoma cell line) were plated and treated with different concentrations of Rh-123 for various times before they were washed and reincubated in fresh medium. The CFU (percentage of control) was determined as described above. The standard error for duplicate samples was 5 percent.



per milliliter (Fig. 1B) and by continuous exposure to 0.2 to 0.5  $\mu\text{g}$  of Rh-123 per milliliter (Fig. 1C).

We also compared the effects of Rh-123 on the CFU of other carcinoma and nontumorigenic cell lines, all of which have been confirmed to be of epithelial origin by studies of immunofluorescence with keratin antibody. The carcinoma cells retained Rh-123 longer than nontumorigenic epithelial cells (4). BSC 1, a nontumorigenic line of monkey kidney epithelial cells (Fig. 2A) and CCL 34, a nontumorigenic dog kidney epithelial line (Fig. 2B) were relatively insensitive to Rh-123. In contrast, CCL 51, a mouse breast carcinoma line (Fig. 2A) and HUT 23, a human lung adenocarcinoma line (Fig. 2B), were very sensitive to the inhibitory effects of Rh-123, with colony formation being reduced to 50 percent of control by continuous exposure to 0.2 to 0.5  $\mu\text{g}$  of Rh-123 per milliliter.

The clonal growth of MCF-7 cells (human breast carcinoma line) and Ehrlich ascites cells (mouse carcinoma line) was also reduced to 50 percent of control after continuous exposure to Rh-123 (0.5  $\mu\text{g}/\text{ml}$ ), whereas the clonal growth of Pt K1 (a nontumorigenic marsupial kidney line) and CRL 1521 (a normal human skin fibroblast line) was greater than 80 percent under similar conditions. However, the clonal growth of all the cell

lines we tested was not reduced by 10 minutes of exposure to 10  $\mu\text{g}$  of Rh-123 per milliliter. Thus, the conditions required for specific staining of mitochondria (1, 2), for measurements of Rh-123 retention (3, 4), and for cell viability assays (3, 7) were not inhibitory to the cells.

Unlike Rh-123, arabinosyl cytosine (Ara-C) and methotrexate (cell cycle-specific anticancer drugs) were not selectively inhibitory for carcinoma cells in vitro (Fig. 2, C and D). Since BSC 1, CCL 34, CCL 51, and HUT 23 had similar doubling times, it appeared that Ara-C and methotrexate inhibited the clonal growth of cycling tumorigenic and nontumorigenic epithelial cells. These results also suggest that the selective inhibition of CFU by Rh-123 was not due to differences in cell-cycle kinetics between carcinoma and nontumorigenic epithelial cells.

The ability of carcinoma lines to retain Rh-123 may have been important in their sensitivity to Rh-123. However, this prolonged dye retention was unlikely to have been the sole mechanism for the selective inhibitory effects since continuous treatment with Rh-123 also resulted in a much greater reduction of CFU in carcinoma cells than in nontumorigenic epithelial cells. Some possible explanations for these observations are (i) that

certain cellular components or organelles (possibly mitochondria) of carcinomas are very sensitive to the inhibitory effects of Rh-123; (ii) that nontumorigenic cells have an active mechanism for excluding Rh-123 from the vulnerable cellular compartment in the face of continued presence of Rh-123 in the culture medium; or (iii) that nontumorigenic cells are capable of inactivating Rh-123. Nonetheless, mitochondria are the likely targets for the inhibitory action of Rh-123 since they selectively accumulate the dye (1-3). Rhodamine-123 may disrupt some mitochondrial functions, such as the translocation of adenosine diphosphate (8), proton ejection (9), or electron transport (10). In previous studies the cytostatic effects of Rh-123 on the L 1210 leukemia line (11) and of rhodamine 6G on the mink fibroblast line CCL 64 (12) were described.

Tumors of the lung, breast, and colon are still the major causes of deaths due to cancer in the United States (13), and new and more selective drugs are needed to combat these tumors. The system we have described in this report would be useful for screening the anticarcinogenic activity of additional rhodamine analogs before they are tested in animals. It would be of interest to determine whether some carcinomas, particularly those that do not retain Rh-123 (4), are resistant to the inhibitory effects of Rh-123. Whether Rh-123 can prolong the survival of mice implanted with carcinomas also remains to be investigated.

SAMUEL D. BERNAL  
THEODORE J. LAMPIDIS  
IAN C. SUMMERHAYES  
LAN BO CHEN

Sidney Farber Cancer Institute  
and Harvard Medical School,  
Boston, Massachusetts 02115

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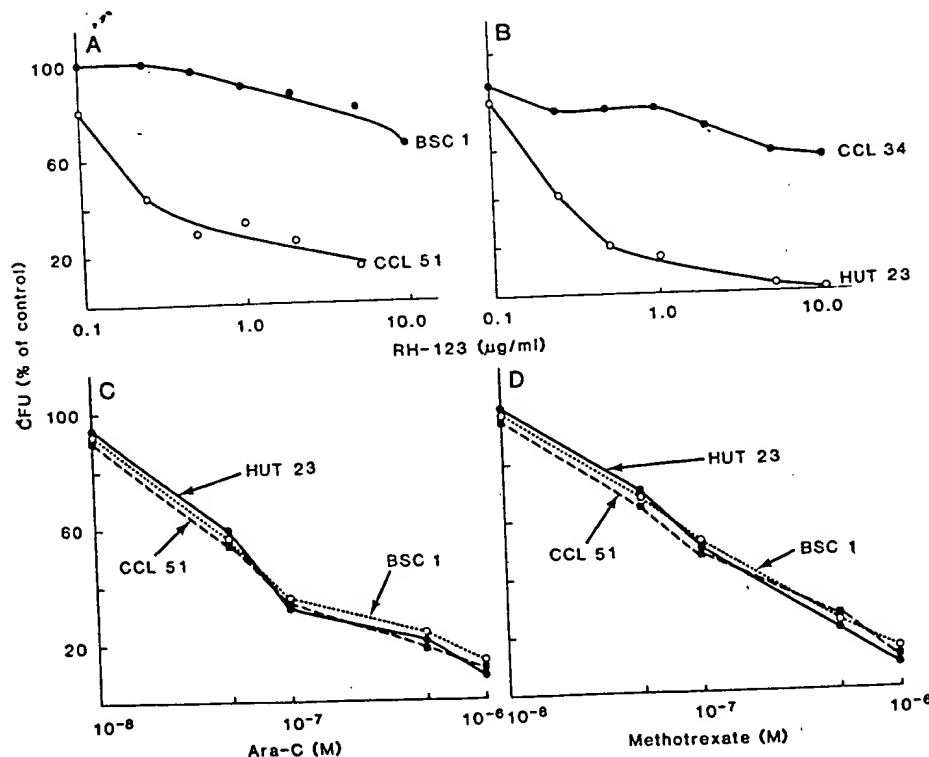


Fig. 2. The effect of Rh-123 on CFU of (A) BSC 1 cells, a nontumorigenic monkey kidney epithelial line, and CCL 51 cells, a mouse breast carcinoma line, and (B) CCL 34 cells, a nontumorigenic dog kidney epithelial line, and HUT 23 cells, a human lung adenocarcinoma line. The effect of (C) Ara-C and (D) methotrexate on CFU of BSC 1, CCL 51, and HUT 23 cell lines.



ex D

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : John A. Arcadi  
Application No. : 08/516,004  
Filed : August 16, 1995  
Title : COMPOSITION AND METHOD FOR  
TREATING PROSTATE CANCER

Grp./Div. : 1200  
Examiner : J. Goldberg

Docket : 28095/RWJ/H29

DECLARATION OF JOHN A. ARCADI

Post Office Box 7068  
Pasadena, CA 91109-7068

Assistant Commissioner for Patents  
Washington, D.C. 20231

Commissioner:

I, John A. Arcadi, declare:

1. I am the inventor named in this patent application.
2. I am the author of the Arcadi 1986 and 1990 references of record in this application, and on which the Examiner relies in rejecting all the claims in this application.

3. My 1986 article discloses the testing of a saline suspension of rhodamine-123 (Rh-123) on rats which had been implanted subcutaneously in the flanks with transplantable R3327-H Dunning rat prostate adenocarcinoma. For that test I wanted to use a solution of 5 mg. of Rh-123 per ml. of solution, but the Rh-123 was in the form of crystalline particles, which would not dissolve entirely in the saline solvent. Accordingly, I stirred the saline suspension vigorously to suspend the Rh-123 particles, filled a hypodermic syringe with the appropriate amount of saline suspension (which contained dissolved Rh-123 and undissolved particles of Rh-123 in the amount of 5 mg. of Rh-123 per ml. of suspension), and within five to ten seconds injected the suspended Rh-123 into the rat being treated. The subcutaneous injection of the saline suspension of Rh-123 described in my 1986 article would be unacceptable for treating patients because the suspension would result in uncertain dosage, and there would be an unknown amount of solubilizing of the Rh-123.

I HEREBY CERTIFY THAT THIS CORRESPONDENCE IS  
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(DATE SIGNED)

*John A. Arcadi*  
*[Signature]*



4. To overcome the problem of using Rh-123 in a saline suspension, I tried an experiment in which I dissolved the Rh-123 in a solution containing 50% dimethylsulfoxide (DMSO) in distilled water. The Rh-123 was sufficiently soluble (5 mg. Rh-123 per ml. of solvent) in the mixture of DMSO and water so as to avoid having to use a suspension. However, I later learned during the course of my work described in my 1995 article that the 50% DMSO mixture with water was unacceptable for administering Rh-123 because it killed some of the mice tested. This is referred to in my patent application on page 6 beginning at line 25 under the section entitled "Toxicity Studies on Mice". Incidentally, in reviewing the description in the application under that heading, I noticed an error in reporting my work. The sentence which begins on page 6, line 29 should read as follows:

"For each solvent there were five groups of five mice each, with the Rh-123 dose per group being 0, 2.0, 7.5, 15, and 20 mg/kg of body weight."

The following sentence should have been inserted after the one set forth above:

"A sixth group of five mice were not given any solvent or Rh-123."

The following table sets forth more fully and accurately the results of the toxicity study referred to in my patent application:

Group No.	No. of Deaths	Treatment
Group 1a	3	20 mg/kg* Rh-123 in 50% DMSO + 50% water**
Group 1b		20 mg/kg Rh-123 in 5% alcohol + 5% glucose*** in water
Group 2a	1	15 mg/kg Rh-123 in 50% DMSO + 50% water
Group 2b		15 mg/kg Rh-123 in 5% alcohol + 5% glucose in water
Group 3a		7.5 mg/kg Rh-123 in 50% DMSO + 50% water
Group 3b		7.5 mg/kg Rh-123 in 5% alcohol + 5% glucose in water
Group 4a		2.0 mg/kg Rh-123 in 50% DMSO + 50% water
Group 4b		2.0 mg/kg Rh-123 in 5% alcohol + 5% glucose in water
Group 5a	2	50% DMSO + 50% water only
Group 5b		5% alcohol + 5% glucose in water only
Group 6		None

\*Mg. of Rh-123 per kg. of body weight

\*\*Distilled water was used for all treatments

\*\*\*The ethyl alcohol was present in the solution in the amount of 5% by volume, and the glucose 5% by weight

Each group in the above table included five mice. As the table shows, three of the five mice in Group 1a died when injected with a dose of 20 mg/kg of Rh-123 dissolved in a mixture of 50% DMSO and 50% distilled water at a concentration of 5 mg. Rh-123 per ml. of solution. One of the five mice in Group 2a died when injected with 15 mg/kg Rh-123 dissolved in a solution of 50% DMSO and 50% distilled water by volume at a concentration of 5 mg. of Rh-123 per ml. of solution.

Two of the five mice in Group 5a died when injected with a solution of 50% DMSO and 50% distilled water containing no Rh-123.

None of the mice died when injected with any of the ethyl alcohol/glucose solutions, even at a dose of 20 mg/kg Rh-123 in a solution of 5 mg. of Rh-123 per ml. of solution, included which 5% ethyl alcohol by volume and 5% glucose by weight in distilled water.

5. The toxicity studies set forth in paragraph 4 above show that the use of the DMSO solutions described in my 1990 article would be totally unacceptable for treating patients.

6. The prostate carcinoma treated in the rats as described in my 1990 article was induced by subcutaneous inoculation with a suspension of Pollard III cells. Thus, none of the experiments described in my 1986 or 1990 articles used Rh-123 to treat ~~autochthonous~~ prostate cancer, that is, prostate cancer which occurs originally in the prostate gland.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date

2/3/97

By

John A. Arcadi

John A. Arcadi

RWJ/clis

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